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A subpopulation of lymphocytes in rheumatoid patients detected by Facb rosettes.

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A SUBPOPULATION OF LYMPHOCYTES IN RHEUMATOID PATIENTS DETECTED BY Facb ROSETTES

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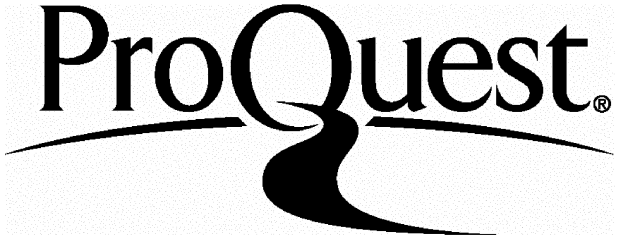

T O M Y P A R E N T S

for their understanding and encouragement

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SUMMARY

A subpopulation of lymphocytes was detected using a novel assay utilising calf erythrocytes sensitised with the Facb fragment of rabbit immunoglobulin G (IgG). This subpopulation was detected in raised percentage numbers in the peripheral blood of rheumatoid patients and was also present in rheumatoid synovial fluid. A cross-sectional study showed that these increased numbers did not map disease activity and were not related to the expression of HLA-DR4. Although normal percentage numbers were present in the peripheral blood of patients with osteoarthritis and ankylosing spondylitis, the elevation is not specific to rheumatoid arthritis since occasionally healthy volunteers showed raised percentages, and percentage numbers also increased transiently following skin testing of sensitised individuals and during viral infection.

The receptor for Facb sensitised erythrocytes (Facb receptor) appears to be a high avidity Fc receptor which is specific for the C_H2 region of IgG and is trypsin and neuraminidase resistant. Phospholipase C also did not diminish Facb receptor expression. The Facb receptor was detected on a small percentage of polymorphonuclear leucocytes and monocytes as well as on lymphocyte surfaces.

Lymphocytes bearing Facb receptors displayed neither T nor B cell markers using conventional techniques. Functional assays showed these cells to be noncytotoxic. A possible role for these cells in immunoregulation is discussed.

CONTENTS

CHAPTER 1

INTRODUCTION

SECTION I

LYMPHOCYTES

This section will be divided into four parts. The first part will be a brief general introduction to the major lymphocyte populations, their origin and role in immune responses. The remaining three parts will describe each of the major lymphocyte populations with particular emphasis on their subpopulations characterised by membrane markers and functional assays.

(i) General Introduction

Lymphocyte Morphology, Origin and Participation in Immune Responses

"The lymphocyte, once considered an end stage cell devoid of known functions, stands at the forefront of new and exciting developments pervading the entire field of biology". So wrote Ritzmann and colleagues in 1973 and continued "it has emerged uncontested as the healthy person's chief guardian against myriads of invading micro organisms, as well as against the enemy that bears from within, the mutant cell with neoplastic potential".

The lymphocyte is indeed a remarkable cell, its functional capacity being masked by its apparent quiescence and uninteresting morphology. Ultimately all lymphocytes derive from pluripotential haematopoietic stem cells. In human peripheral blood, lymphocytes comprise 20-40% of the differential leucocyte count and range in diameter from 6-25 μm. The majority of these cells are small

lymphocytes (6-9μm) while a few may be intermediate (9-12μm) or large (12-25μm); their life span varies from a few days to several months for recirculating small cells. All three size types have a dense nucleus surrounded by a basophilic cytoplasm, due to the ribosomes present, and few organelles. The larger cells have more cytoplasm and may have a nuclear cleft.

Since Gowans (1962) first showed that small lymphocytes were involved in graft versus host reactions and gave rise to large pyroninophilic cells which can divide, evidence for lymphocyte recirculation and their participation and co-operation in immune responses has expanded (Sell and Gell, 1965; Roitt et al, 1969; Greaves, Torrigiani and Roitt, 1969; Parrott and De Sousa, 1971; Sprent, 1977; Howie, 1978). The immune response appears to be macrophage dependent (Askonas and Roelants, 1974; Dupont Guerry et al, 1978; De Vries et al, 1979; Nathan, Murray and Cohn, 1980; Rosenthal, 1980; Martinez-Alonso, Bernabe and Diaz-Espada, 1980) and genetically restricted (Winchester, Wernet, Dupont and Kunkel, 1975; Barnstable and Jones, 1977; Burakoff et al, 1980; Rich et al, 1980). It is generally accepted that there are two major arms of the immune response (Roitt et al, 1969):-

(a) humoral immune responses generated by B lymphocytes which can synthesise antibody

(b) cell-mediated immunity dependent on T lymphocytes

T or thymus-derived lymphocytes are of two types, being either cortical or medullary thymus-derived lymphocytes. In the mouse, the former have been characterised as small cortisone sensitive lymphocytes with a rapid turnover having a high content of Thy-1 antigen

and a low content of H2 antigen, while the latter have a slower turnover, more H2 antigen and are cortisone resistant. It has been demonstrated that neonatally thymectomised mice cannot evoke graft versus host reactions, do not express delayed-type hypersensitivity and show minimal responses to certain (T-dependent) antigens (Loor and Roelants, 1977).

B lymphocytes were so named after the avian Bursa of Fabricius, a cloacal lymphoid organ. Bursectomised chickens were found to have reduced levels of serum immunoglobulin, few plasma cells and depleted populations of surface immunoglobulin bearing small lymphocytes (Loor and Roelants, 1977). The bursal equivalent in mammals is a highly contentious element; bone marrow or gut-associated lymphoid tissue is favoured by most investigators. It is likely that both are involved. The thymus and Bursa of Fabricius are primary lymphoid organs in which lymphopoiesis is intense, independent of antigenic stimulation and prominent before birth. Bone marrow may also fall into this category.

The secondary lymphoid organs contain a mixture of T and B cells and lymphopoiesis is relatively slow; the lymphocytes are not sessile but migrate continuously via the blood stream and lymph. The spleen, lymph nodes and certain regions of the alimentary tract (tonsil; Peyer's patches of the ileum; appendix) are all examples of secondary lymphoid organs.

Lymphocyte Separation and Identification

The most commonly employed method of lymphocyte separation is

by density flotation on ficoll/diatrizoate (Bøyum, 1968). More recently polyvinylpyrrolidine-coated silica (PVP-Silica; Percoll) has been shown to be a useful separation medium for lymphocytes (Kurnick and co-workers, 1979).

T and B cell identification and separation is of prime importance for any functional definition of lymphocyte subpopulations. Morphologically B and T cells are identical when viewed by conventional light microscopy. However using the scanning electron microscope, Polliack and colleagues (1973) showed that B cells have surface projections ("hairy cells") whereas T cells present a bland flat surface. Further work by Polliack and co-workers (1974) demonstrated that "hairy cells" resulted from cell activation. This work is now thought to be artefactual and more conventional methods of detection are employed. A number of surface markers have been accepted to differentiate between T and B lymphocytes (WHO/IARC, 1974). It is necessary to test for as many markers as possible on a given cell since it is increasingly evident that within the T/B groups there are many subclasses.

A wide variety of surface markers are available and these will be presented in detail in the next sections. The application of these techniques has resulted in the identification of a third population of null lymphocytes (Frøland and Natvig, 1973). Null lymphocytes lack conventional T and B lymphocyte surface markers but bear high avidity Fc receptors. These cells will be discussed in detail in Chapter 1, Section I (iv). Lymphocytes may also be classified by their functional characteristics (Jondal, Wigzell and Aiuti, 1973). Thus K and NK lymphocytes have been described which

mediate specific cytotoxic reactions (Chapter 1, Section I (ii) and (iv)).

(ii) Thymus-Derived (T) Lymphocytes

This section will consist of a short introduction, listing T cell immune responses, and a brief report of mouse T cell identification and functional characterisation. Human T lymphocyte subpopulations, defined by surface markers, and the functions ascribed to such subpopulations will be described in detail.

Introduction

As previously stated, T lymphocytes are mediators of cellular immune responses (Roitt et al, 1969). T lymphocytes are known to produce soluble non-specific factors following interaction with antigen; these non-immunoglobulin factors have been termed lymphokines (Dumonde et al, 1969). Soluble factors have also been demonstrated following interaction with mitogens (Maini et al, 1969). Delayed type hypersensitivity has been attributed to a T cell subclass (Huber et al, 1976) and, more recently, T lymphocytes have been implicated in the response of B cells to certain mitogens and to antigenic stimulation. T lymphocytes which augment B cell responses are termed helper T cells and those which diminish B cell responses are T suppressor cells (Moretta et al, 1976; 1977; Lipsky, Ginsberg, Finkelman and Ziff, 1978; Moretta, Mingari, Moretta and Cooper, 1979; Johnsen and Madsen, 1979c; Dillner-Centerlind et al 1980). Cytotoxic T cells have been described and are generated during mixed lymphocyte reactions (Webb, Mason and Williams, 1979;

Palacios et al, 1980). Mitogen-induced, antibody dependent and spontaneous cellular cytotoxicity have been attributed to T lymphocytes by some workers. These will be described in detail later in this section.

Identification and Functional Characterisation of Murine T Cells

The expression of the Thy-1 (theta; θ) antigen, first recognised by Reif and Allen (1964) is the most characteristic feature distinguishing T cells in mice. Functional studies have been facilitated by the use of antisera to certain lymphocyte antigens. The Ly (lymphocyte) subclasses were defined by Shiku and colleagues (1975) and a Ly 1 negative (Ly 1-), Ly 2,3 positive (Ly 2,3+) cytotoxic cell was characterised. Cantor and Boyse (1975 (a); 1975 (b)) showed differential expression of Ly 1, Ly 2 and Ly 3 on peripheral blood Thy 1 positive mouse cells, about 50% having all three Ly antigens (Ly 1, 2, 3+), 33% Ly 1+ and 7% Ly 2,3+. Helper T cells were shown to be Ly 1+ while Ly 2,3+ cells generated cytotoxic activity. Vadas and co-workers (1976), investigating phenotypes of T cells involved in delayed type hypersensitivity (DTH) and suppression, confirmed that T cells responsible for DTH and helper function were Ly 1+2- while cytotoxic and suppressor T cells were Ly 1-2+. The same workers showed that immune-associated (Ia) antigens produced by the I region of the mouse major histocompatibility complex (H2) were present on suppressor cells while DTH responsive cells were Ia negative. The presence of these differentiation antigens has made it possible to investigate more easily cell-mediated immunity in the mouse (Leclerc and Cantor, 1980 (a); 1980 (b)).

Identification of Human T Cells and T Cell Subpopulations

Human T cell identification has proved more difficult than murine systems. A number of surface markers have been described which can be used for human T cell identification.

E Rosettes

Jondal, Holm and Wigzell (1972) described a population of human lymphocytes forming spontaneous rosettes with sheep erythrocytes (SRBC). They showed that rosette formation occurred only with living cells and was temperature and cation dependent; inhibition of rosette formation occurred when the reaction mixture was warmed to 37°C and when sodium iodoacetate (which blocks glycolysis) or ethylene diamine tetra acetic acid (EDTA) was added. Trypsinised lymphocytes did not form SRBC rosettes but this ability was restored after culture. Lymphocytes forming these spontaneous 'non-immune' E rosettes were later confirmed to be thymus-derived (Wybran, Carr and Fudenberg, 1972; Wybran and Fudenberg, 1973; Jondal, 1976).

Yu (1975) characterised two populations of T lymphocytes by their ability to bind SRBC, defining them as early or active rosette-forming cells (RFC) and late or inactive RFC. Early rosettes are formed immediately after sedimentation while late rosettes require incubation at 4°C for optimal formation. Chisholm and Tubergen (1976) using varying SRBC/lymphocyte ratios were unable to identify discrete populations of T cells. Further modifications of the original method have appeared; prior treatment of the erythrocytes

with the sulphydryl reagent 2-aminoethylisothiouronium bromide (AET), or with neuraminidase enhances binding resulting in increased numbers of more stable rosettes (Kaplan and Clark, 1974).

The SRBC receptor is still the most widely used T cell marker although attempts at characterisation of the receptor have, until very recently, been minimal (Pyke, Rawlings and Gelfand, 1975). Kamoun and co-workers (1981), using a murine monoclonal antibody, have identified a protein on human T lymphocytes which is associated with the E rosette receptor. Suomalainen, Goldsby, Osborne and Schrøder (1980) using mouse/human hybrid T cells have shown that expression of the SRBC receptor is genetically coded by the human chromosome 6. A human homologue of Thy-1 has also been characterised (Ades, Zwerner, Acton and Balch, 1980).

Helix Pomatia (HP) Receptors

Hammarstrøm and co-workers (1973) described another useful marker for T lymphocytes. Neuraminidase treated lymphocytes express a receptor for Helix pomatia (vineyard snail) A haemagglutinin. Affinity chromatography can be used to isolate enriched T cell populations (Hellstrøm et al, 1976). The Helix pomatia receptor positive (HP+) cells are mainly T cells although a small population ($\sim$10%) do not form E rosettes.

Fc Receptors

In 1975, Basten and associates showed the presence of a receptor for the Fc portion of immunoglobulin G (IgG) on a subpopulation of

murine T cells and concluded that IgG Fc receptor (FcR) bearing T cells represent a subpopulation of cells within the thymus and the secondary lymphoid tissue. In man, two distinct T cell subpopulations have been defined on the basis of IgM or IgG Fc receptor expression. Thus E rosette positive cells bearing receptors for IgG are termed T_γ (T gamma) cells (Ferrarini et al, 1975) and those T cells with IgM Fc receptors are T_μ (T mu; Moretta et al, 1975). A small population of T cells exist having neither receptor.

Ia Antigens

Ia antigens were first recognised on murine B lymphocytes and subsequently demonstrated on human lymphocytes (Lunney, Mann and Sachs, 1979). Fu and co-workers (1978) and Metzgar and associates (1979) identified Ia antigens on activated T cells in man.

Antisera

Antisera have been raised against human T lymphocytes in an attempt to recognise an equivalent to the murine Ly series. Various approaches have been made. Heteroantisera have been produced against T lymphoblastoid cell lines (McMahon Pratt, Schlossman and Strominger, 1980; Niaudet and Greaves, 1980), human Ia-like antigens of the HLA-D region of chromosome 6 (Broder, Mann and Waldmann, 1980) and T lymphocyte Fc receptors (Cunningham-Rundles et al, 1980). Early work, using rabbit antisera raised against human lymphocytes and rendered specific for T cells by adsorption, characterised two populations of T lymphocytes, the TH1 and TH2 subsets (Evans et al, 1977; 1978).

Monoclonal Antibodies

A recent step forward in the characterisation of human T lymphocytes has been the production of monoclonal antibodies directed against cell surface antigens. Köhler and Milstein (1975) first successfully cloned hybrid cells producing a specific monoclonal antibody, and since then many T cell specific monoclonal antibodies have been prepared (Reinherz and Schlossman, 1980). Thus OKT1 and OKT3 are specific for all peripheral blood T cells while OKT4 recognises T helper cells and OKT5 reacts with suppressor/cytotoxic cells (Reinherz, Kung, Goldstein and Schlossman, 1980). Ia positive cells react specifically with the monoclonal antibody OKI1. A list of monoclonal antibody specificities is given in Table 1.1.

Functional Characterisation of Human T Cell Subpopulations

Functional characterisation of human T cell subsets has been attempted using methods involving skin testing, antigenic or mitogenic stimulation and measurement of the cytotoxic capacity of various subpopulations.

Delayed Type Hypersensitivity (DTH)

The active E rosette test has been adapted by many workers; Felsburg and Edelman (1977) reported elevated levels of active E rosette forming cells 24-72 hours after intradermal skin testing of sensitised individuals. Skin-test negative individuals showed no change in the level of active E rosette forming cells. Thus

Table 1.1

MONOCLONAL ANTIBODIES EMPLOYED IN THE CHARACTERISATION OF HUMAN T LYMPHOCYTE SUBPOPULATIONS

MONOCLONAL ANTIBODY	SPECIFICITY
OKT 1	Peripheral blood total T population
OKT 3	Peripheral blood total T population
OKT 4	Peripheral blood helper/inducer T population
OKT 5	Peripheral blood cytotoxic/suppressor T population
OKT 6	Thymocytes
OKT 8	Peripheral blood cytotoxic/suppressor T population
OKT 9	Thymocytes
OKT 10	Thymocytes
OKI 1	Ia antigen positive populations
OKM 1	Monocyte/myeloid population

the assay is specific and appears to correlate with delayed type hypersensitivity. A group of Japanese workers (Nagaoki et al, 1978) demonstrated a transient increase of Tγ cells in tuberculin sensitive individuals following skin testing with a purified protein derivative of tuberculin (PPD); this increase was observed 36-48 hours following inoculation. No significant change was noted in skin test negative individuals. Yu and colleagues (1980), using tetanus toxoid, showed increased levels of Ia positive T cells in sensitised individuals within two days of immunisation. As previously stated, Ia expression is associated with T cell activation (Fu et al, 1978; Metzgar et al, 1979).

In Vitro T Lymphocyte Activation by Mitogens and Antigens and in Mixed Lymphocyte Cultures

Functional characterisation of active E rosetting cells, described by Hokland and Heron (1979), compared their in vitro response to antigen and mitogens. Active E rosette forming cells (RFCs) responded significantly better to PPD and allogeneic cells than did late non-active RFCs. The converse was true with phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), when the non-active (low-avidity receptor) T cells gave higher responses and were better stimulators in a mixed lymphocyte reaction. Monocyte dependence was shared by both subsets. The finding that early T cells are sensitive to PPD stimulation and are better responders in mixed lymphocyte culture (MLC) provides further support that this subpopulation is involved in cell mediated immunity. Characterisation of subsets of T cells, in terms of SRBC affinity, was

attempted by Sasaki and co-workers (1975), using two different buffering systems. They found an increased PHA responsiveness in one subset but their results cannot be directly correlated with the early and late rosettes described by Yu (1975).

Using rosette techniques, Moretta and colleagues (1976) isolated $T\gamma$, $T\mu$ and $T\gamma$ depleted (i.e. $T\mu$ enriched) subpopulations of lymphocytes and compared these with a total T cell population. They demonstrated similar Con A dose response curves in all the populations tested while the PHA dose response curves showed a significant reduction in the response of $T\gamma$ cells relative to total T cells. $T\gamma$ depleted populations showed the greatest variability in individual patterns of PHA responsiveness. It was thus concluded that a normal PHA response requires synergism between the two T cell subsets. Moretta, Webb, Grossi, Lydyard and Cooper (1977), using PWM, reported that helper activity was confined to the $T\mu$ population while the $T\gamma$ population, after interaction with IgG immune complexes (termed modulation) during isolation, suppressed the generation of plasma cells when added to helper T cells and B cells together with PWM. No such suppression was seen using $T\mu$ depleted (i.e. $T\gamma$ enriched) populations where no interaction with immune complexes had occurred. Dependence on modulation for expression of suppressor activity was confirmed using T lymphocyte clones (Canonica et al, 1980).

Functional characterisation has been attempted using the TH1 and TH2 subsets previously described. Thus Evans and colleagues (1977) showed that TH1 positive T cells comprised 50-60% of all T cells, proliferated in the MLC and generated lymphokine but did not proliferate in response to certain soluble antigens (mumps,

PPD, tetanus toxoid). Conversely, the TH1 negative subset neither responded in the MLC nor produced lymphokine but mounted a good proliferative response to the specific antigens. The TH2 positive subset, which is inactive in MLC, mediates cytotoxic and suppressor functions (Evans et al, 1978; Reinherz and Schlossman, 1979).

T Cell Mediated Cytotoxicity

A wealth of literature is available on the cytotoxic potential of human lymphocytes. Thus, E rosette positive lymphocytes have been implicated in mitogen-induced (PHA) cellular cytotoxicity (MICC), antibody-dependent cellular cytotoxicity (ADCC) and natural or spontaneous cell mediated cytotoxicity (SCMC). Much controversy has arisen concerning the identification of killer (K) cells, which mediate ADCC, and natural killer (NK) cells, responsible for SCMC. Some workers have ascribed K and NK cells to the same population (De Landazuri et al, 1979) while others have successfully separated the two populations (Neville, 1980).

Waller, Campbell and MacLennan (1976), using columns coated with albumin or immune complexes (IgG - anti IgG), demonstrated that two populations of lymphocytes are involved in MICC, one dependent and the other independent of Fc receptors. A large proportion of T_γ cells were retained on the IgG-anti IgG column (approx. 20%) and these were presumed to be responsible for the observed drop in MICC ($\frac{2}{3}$ activity removed). Both ADCC and SCMC were completely abolished by removal of FcR positive cells. Perlmann, Perlmann, Pape and Halldén (1976) observed similar effects using ovalbumin-anti ovalbumin affinity columns; the E

rosette positive fraction showed some depletion of Fc receptor positive T cells after passage over the immune complex column and the unbound population had very low K cell activity.

Direct measurements of MICC confirmed the dependence on two different subpopulations bearing Fc receptors (Cordier, Samarut and Revillard, 1978). Both populations were C3 receptor negative but only one carried a T cell marker (E rosette positive); the other was non-T in nature. Other investigators have demonstrated the release of a factor by PHA stimulated lymphocytes which augments E and EA rosette formation (Agbata and Kirkpatrick, 1979; Kubota, Yoshida, Shinomiya and Mikawa, 1980). This may provide indirect evidence for T_γ mediation of MICC and the soluble factor may be important in the mediation of other cellular immune responses.

In 1976, Nelson and co-workers attempted to differentiate the effector cells in MICC and ADCC in terms of their target specificity. Using chicken erythrocytes (CRBC) and Chang liver cells, they concluded that MICC was directed against both target cells by a non-FcR bearing T cell whilst T lymphocytes did not mediate ADCC against either CRBC or Chang cell targets.

Initial direct evidence for T cell co-operation in ADCC was supplied by West, Boozer and Herberman (1978) who showed that ADCC activity was confined to the non-active E (low avidity SRBC receptor) rosette population. Jónsdóttir and co-workers (1979) demonstrated K cell activity in both active and non-active E rosette forming cells. In parallel with these observations, Moretta, Mingari and Romanzi (1978) showed a loss of IgG FcRs from T_γ cells modulated

with immune complexes and reported that T_γ depleted subpopulations and isolated cultured T_γ cells (i.e. immune complex modulated) no longer carried out ADCC. Pichler, Lum and Broder (1978) also observed a transition of receptor expression demonstrating that T_γ cells can express Fc receptors for IgM after immune complex modulation; no functional studies were performed. Extending this work, Pape, Moretta, Troye and Perlmann (1979) confirmed the loss of ADCC activity after immune complex modulation, but reported that a significant part of the NK activity was retained. Similar experiments by Shen, Lydyard, Penfold and Roitt (1979) demonstrated T_γ mediation of ADCC and reported that this activity could not be ascribed to contamination by monocytes or neutrophils. However, Hammarström (1979) showed that cultured human monocytes could be induced to kill K562 target cells when activated by supernatants of autologous lymphocytes stimulated with bacteria (lymphokines).

Using Helix pomatia Sepharose, Pape, Troye and Perlmann, (1979) demonstrated NK activity in both HP+ and HP- subpopulations. The HP+ lymphocytes were thought to be T cells. Experiments using whole lymphocyte populations showed that NK activity can be further subdivided since some of the activity is lost after adding Fab fragments of rabbit anti human IgG. The authors suggest that antibody dependent natural cytotoxicity may occur (Pape, Troye, Axelsson and Perlmann, 1979). However if virus-infected target cells were used in the assay, no immunoglobulin specific NK activity was observed (Härfast, Andersson and Perlmann, 1978). It was concluded that the elevated cytotoxicity observed was probably spontaneous, virus-dependent and mediated, not through antibodies, but by some non-immunological agent, perhaps interferon; interferon is

known to boost NK activity in vitro (Ortaldo et al, 1980). Fc-receptor modulation had no effect on virus-dependent cytotoxicity indicating that a functionally intact FcR is not required for cytolysis in this system (Härfast, Andersson, Alsheikhly and Perlmann, 1980). The recent production of a cloned line of natural killer cells may help to unravel some of the questions concerning the role of these cells in immunity (Dennert, 1980).

T Lymphocyte Characterisation by Surface Markers and Functional Assays - Technical Difficulties

It must be stressed at this point that many of the isolation procedures used to obtain "pure" T cell subsets may result in alteration or elimination of functional capacity. Modulation with immune complexes has been described previously; this causes an increase in suppressor activity and elimination of ADCC (Moretta, Webb, Grossi, Lydyard and Cooper, 1977; Moretta, Mingari and Romanzi, 1978). Neuraminidase treatment, employed prior to affinity chromatography on Helix pomatia Sepharose, has been shown either to stimulate NK and K cell activity (Kay et al, 1977) or to have no significant effect (Schulof, Fernandes, Good and Gupta, 1980). Ammonium chloride treatment, used in the isolation of T_γ cells, has been shown to depress the cytotoxic response when measured using a 4 hour assay but this capacity was restored after incubation for 24 hours (Kay et al, 1977). Thus ammonium chloride is only detrimental in the short term.

Probably the most exciting recent development in the analysis of lymphocyte subpopulations has been the introduction of monoclonal

antibodies to specific surface antigens. Inevitably, comparisons must be made between cells separated using these antibodies and cells characterised by previously accepted markers. Reinherz and co-workers (1980) compared T lymphocyte subpopulations defined by Fc receptors and monoclonal antibodies; the results obtained were interesting but disappointing in that, other than a slight enrichment of T_{μ} cells within the OKT4 subset, there was little correlation between T cell subsets defined by the two techniques. A large number of cells in the T_{γ} population were Ia negative and reacted with OKM1, suggesting that many so named T_{γ} cells are of monocyte/myeloid origin. This may again be due to preparative procedures since neuraminidase enhances binding of sheep erythrocytes but may reduce specificity. Both T_{γ} and OKT5+ cells can be induced to suppress following activation by Con A; however T_{γ} cells effect SCMC and ADCC which is not a property of unactivated OKT5+ cells. The authors infer that the T_{γ}/OKM1 subset may be natural killer cells.

It is apparent that even after twenty years of research, the role of T lymphocyte subsets in immune responses remains unclear. However, much progress has been made and the present techniques available will, without doubt, facilitate definition of these subsets.

Table 1.2 summarises the work presented in this section concerning functional aspects of human T cell subpopulations with respect to surface markers.

(iii) Bursa Derived (B) Lymphocytes

This section will essentially follow the same pattern as the

Table 1.2

SURFACE MARKERS DETECTED ON FUNCTIONAL

HUMAN T LYMPHOCYTE SUBPOPULATIONS

FUNCTION*		SURFACE MARKER								
		Early E	Late E	$Fc_{\gamma}R$	$Fc_{\mu}R$	Ia	TH1	TH2	OKT4	OKT5
HELP					+				+	
SUPPRESSION				+				+		+
DTH		+		+		+				
MLC	Responder	+					+			
	Stimulator		+						+	
MICC				+/-						
ADCC		+/-	+	+				+		+
SCMC				+						
ACTIVATION	PPD	+								
	PHA		+	synergism ⟷						
	Con A		+	+	+					
	PWM		+							

* For abbreviations see text

T lymphocyte section in that it will consist of a short introduction followed by a report of B cell surface markers and their interactions. Finally attempts to characterise B cell subpopulations will be accounted.

Introduction

The primary role of B lymphocytes in immune responses is the synthesis of specific antibody. Surface immunoglobulin (sIg) forms an integral part of the B cell membrane and this can act as an antigen binding receptor although triggering probably involves more signals than this single interaction. Four cells have been implicated in the initiation and regulation of antibody synthesis (Fagraeus, 1981). A simplified scheme of events may be as follows. The macrophage takes up antigen and presents it to the T helper cell which receives both the antigen and products of the immune response genes of the major histocompatibility complex. The T helper cell then passes these same or similar products to the B cell which is triggered following antigen recognition. The B cell produces antibody with the same specificity as the surface immunoglobulin recognition molecules and differentiates into a plasma cell. Receptor immunoglobulin recognition molecules are lost during differentiation (Abdou, Alavi and Abdou, 1976). The fourth cell, the suppressor cell, triggered by macrophage produced factors, acts on the T helper cell and perhaps the B cell to regulate antibody synthesis. T suppressor cells have been described previously (Chapter 1, Section I (ii)). B suppressor (Zubler et al, 1980) and macrophage suppressor cells (Denman, 1979) have also been reported. The presentation of antigen by macrophages may be more complex. It

has been postulated that a genetically restricted factor (GRF) released by the macrophage acts on an early T cell (Ly phenotype 1, 2, 3) which in turn enlists the T helper cell (Ly 1) by the release of soluble factors (Howie, 1978).

B cells can be induced to lose sIg by incubation with anti-immunoglobulin sera and thus their antigen recognition site is lost (Raff et al, 1975). Antigen recognition is important to avoid the production of autoantibodies. Nossal and Pike (1975) proposed that during development B lymphocytes contact self antigens and thus achieve immunological unresponsiveness or tolerance to self. Auto-immune disease (e.g. rheumatoid arthritis) presents a disturbance of the self/non-self discriminating mechanism.;

Non-T Lymphocyte Surface Markers

Much of the early work on B cell characterisation was performed on non-T cells. Mouse non-T cells specifically express the allo-antigen Ly 4.2 (McKenzie and Snell, 1975).

Surface Immunoglobulin (sIg)

The presence of surface immunoglobulin (sIg) is probably the most widely accepted B cell marker. Raff, Sternberg and Taylor (1970) demonstrated sIg on mouse lymphocytes; these cells were subsequently shown to be non-T cells (Rabellino, Colon, Grey and Unanue, 1971). Papamichail, Brown and Holborow (1971) confirmed the presence of sIg on human lymphocytes and Frøland and Natvig (1972 a) described a lack of sIg positive lymphocytes in hypogammaglobulinaemia, implying

that these lymphocytes were of B cell lineage. Further work by Frøland and Natvig (1972 b) showed class, subclass and allelic exclusion of sIg on human non-T lymphocytes, the dominant class being IgM and the dominant subclass of IgG being IgG2. Taylor and co-workers (1971), using mouse non-T lymphocytes, demonstrated that antibody interaction caused redistribution of sIg to one pole of the cell (capping) and subsequent pinocytosis of immunoglobulin molecules. These authors suggested that cap formation and pinocytosis may trigger lymphocyte transformation (blastogenesis) or tolerance induction.

Complement Receptors

Non-T cells were shown to possess a receptor for the third component of complement, C3 (Bianco, Patrick and Nussenweig, 1970). Ross, Polley, Rabellino and Grey (1973) characterised two different complement receptors on human lymphocytes, the C3b or immune adherence receptor and the C3d receptor. They are antigenically distinct, cap independently and have specificities for different regions of C4 or C3 molecules (Ross and Polley, 1975; 1976).

Fc Receptors

Binding of both heat aggregated human IgG, agg.G (Dickler and Kunkel, 1972; Dickler, 1976 a) and antigen complexed IgG (Basten, Miller, Sprent and Pye, 1972) was demonstrated by non-T cells. Binding was dependent on an intact Fc portion of the immunoglobulin and thus the term Fc receptor (FcR) was applied. Soluble molecular antigen-antibody complexes (immune complexes) or cellular antigen-

antibody complexes (EA rosettes) have been used extensively in the study of B lymphocyte Fc receptors (Brown and Greaves, 1974; Johnsen and Madsen, 1979 a). Dickler (1974) demonstrated binding of both immune complexes and aggG by the same receptor on human B cells.

B Lymphocyte Identification - Technical Problems and the Presence of a Third Lymphocyte Population

In 1973, Ross, Rabellino, Polley and Grey reported that not all C3 receptor positive lymphocytes were sIg positive and that almost all sIg negative cells were E rosette positive. Frøland, Natvig and Michaelsen (1974) suggested that binding of immunoglobulin by B lymphocytes may be independent of Fc receptors. It soon became evident that not all non-T lymphocytes were bursa-derived but that a population of non-T, non-B lymphocytes existed (Frøland, Wisløff and Michaelsen, 1974; Kurnick and Grey, 1975; Natvig and Frøland, 1976) and also lymphocytes expressing B and T cell markers (Van Boxel and Rosenstreich, 1974; Dickler, Adkinson and Terry, 1974). The Fc and C3 receptors could not be regarded as specific B cell markers.

Methods of B lymphocyte separation and detection have been intensively studied and technical problems were discovered (Winchester, Fu, Hoffman and Kunkel, 1975). Thus, two populations of sIg positive human lymphocytes were described (Lobo, Westervelt and Horwitz, 1975). The non-T population was shown to contain lymphocytes with cold reactive FcRs for cytophilic immunoglobulin (Horwitz and Lobo, 1975) and therefore non-T lymphocytes were

described as having membrane incorporated (B cells) or membrane labile (null cells) immunoglobulin (Lobo and Horwitz, 1976). Kumagai and co-workers (1975) investigated the membrane incorporated sIg on non-T cells. They demonstrated that incubation at 37°C or acid treatment of lymphocytes resulted in the loss of surface IgG and IgA but had no effect on IgM. This was confirmed by Pettersson, Meldstedt and Holm (1978) who showed that incubation and washing of lymphocytes at 37°C decreased the total sIg and surface IgG measured but had no effect on surface IgM or IgD. Ross, Winchester, Rabellino and Hoffman (1978) showed that all sIgM positive lymphocytes were FcR positive and expressed both C3b and C3d complement receptors. Alexander and Henkart (1978) concluded that most human peripheral B lymphocytes do not have easily detectable Fc receptors and most FcR bearing cells do not have B cell markers.

B Lymphocyte Surface Markers

Surface immunoglobulin, C3 and Fc Receptors

Human B lymphocytes bear surface membrane incorporated IgM and C3 and Fc receptors (see above). B cell $Fc_{\gamma}Rs$ are sometimes difficult to detect (Alexander and Henkart, 1978; see also Chapter 1, Section III).

The demonstration of Fc_{μ} receptors on T lymphocytes (reported in the previous section) prompted work using B lymphocytes. Human peripheral blood Fc_{μ} positive B cells (sIgM positive) were found only occasionally but 30% of tonsil B lymphocytes expressed Fc_{μ} receptors (Pichler and Knapp, 1978). Pichler and Broder (1978), using B cell (sIg and C3 positive) enriched human peripheral blood

lymphocytes demonstrated Fcμ receptors on all B lymphocytes following incubation at 37°C. Preud'homme and colleagues (1979) investigated culture conditions and found that these had some effect on Fcμ receptor detection; a second culture in a different medium was often needed to detect Fcμ receptors on lymphoblastoid cell lines.

Ia Antigens

The immune-associated (Ia) antigens are products of the I region of the murine major histocompatibility complex (H2). Mouse allo-antisera have been prepared and shown to cross react with human lymphocytes (Lunney, Mann and Sachs, 1979). Thus human Ia-like antigens were shown to exist. Human B lymphocyte alloantigens have been studied (Johnson, Ward and Amos, 1977) and HLA-D locus related (HLA-DR) antigens have been shown to be analagous in tissue distribution and structure to murine Ia antigens (Snary et al, 1977).

Virus Receptors

Receptors for Epstein Barr virus (EBV), a member of the herpes virus group, have been detected on B lymphocytes (Jondal and Klein, 1973) and this marker is specific for B lymphocytes (Greaves, 1976). The response to EBV was shown to be T independent (Kirchner et al, 1979) although other workers (Biberfeld et al, 1980) have shown that PPD and PWM (T dependent activators) induce viral antibody production by B cells.

Other Surface Markers

Other methods of B cell identification include rosette

formation using mouse erythrocytes (Bertoglio, Laldjim and Doré, 1979) and the use of hybridoma antibodies (Beckman et al, 1980; Nadler et al, 1981).

Surface Marker Interactions

Two groups of workers suggested that Ia antigens and Fc receptors on mouse B lymphocytes were either identical or closely related (Dickler and Sachs, 1974; Basten, Miller and Abraham, 1975). Inhibition of mouse B lymphocyte Fc receptors has been attempted using IgG and $F(ab')_2$ fragments of anti-Ia (Dickler,Arbeit and Sachs, 1975), and antisera to H-2D and H-2K (non Ia producing loci of the mouse H-2 complex), Ly 4.2, mouse immunoglobulin (Halloran and Schirrmacher, 1977), B_2 microglobulin (Grey, Anderson, Huesser and Kurnick, 1975) and Thy-1 (Schirrmacher, Halloran and David, 1975). Both IgG and $F(ab')_2$ antibodies to Ia antigens inhibited EA rosettes but antibody against irrelevant H2 antigens or against T cell antigens failed to inhibit EA rosette formation. This strengthens the view of Dickler and Sachs (1974) and Basten, Miller and Abraham (1975). Other workers have shown that FcRs and Ia antigens are independent entities on the B cell membrane (Schirrmacher and Halloran, 1975). In man FcRs and DRw antigens have also been demonstrated to be independent of each other (Soulillou and Peyrat, 1979). Interaction may be dependent on a ligand-binding mechanism (Rieber and Wernet, 1977). An interaction between Ia antigens and C3 receptors has also been described (Schirrmacher et al, 1976).

Associations between sIg and FcRs have been investigated.

Under normal circumstances these receptors are independent (Ramasamy and Lawson, 1975) but under certain conditions (e.g. anti-Ig-induced redistribution) capping of sIg results in co-capping of FcRs suggesting that some interaction takes place (Forni and Pernis, 1975). This observation was confirmed by Unanue and Abbas (1975) but the reverse phenomenon (i.e. co-capping of sIg by fluoresceinated antibody bound to FcRs) was not observed and capping of sIg with concomitant capping of FcR did not produce alterations in Ia distribution. This latter observation opposes the view of Dickler and Sachs (1974). In contrast, Scribner, Weiner and Moorhead (1977) have shown that activated cells cannot co-cap. Recently Dickler and Kubicek (1981) have demonstrated a specific interaction between ligand-bound surface IgM and ligand-bound Fc Rs on B lymphocytes; their results also suggest that whenever IgM is involved in the response to an immunological stimulus, the $Fc_{\gamma}R$ is also involved.

Most workers agree that sIg, C3 and Fc receptors and Ia antigens are independent entities on the B cell membrane but may interact following antigen binding (Parish and Hayward, 1974; Unanue and Abbas, 1975).

B Cell Subpopulations

Subsets of B cells have proved difficult to isolate. Several workers have attempted to subdivide B cells in terms of their response to mitogen. Some B cell mitogens do not only induce DNA synthesis and mitosis but may activate the cells to synthesise immunoglobulin. Thus B cell mitogens are more correctly termed

polyclonal activators (PCA). Ringdén and Møller (1976) have described three types of B cell in terms of response to PCA; these are non-responders, B cells which respond by proliferation but not immunoglobulin production and those which proliferate and become high rate antibody synthesising cells. The same workers showed that rabbit antibodies to β_2 microglobulin were mitogenic but antibody synthesis occurred only with spleen and tonsil cells and not peripheral blood lymphocytes, suggesting different subsets. The response was found to depend on the stage of ontogenesis. The protein β_2 microglobulin (mol.wt. $\sim$ 12,000) was known to display a partial homology with the constant domains of IgG1 heavy chains and to be associated with HLA antigens on the B cell membrane (Neauport-Sautes, Bismuth, Kourilsky and Manuel (1974). It is now known that β_2 microglobulin forms an integral part of the HLA-A and HLA-B antigens.

Beverley (1977) has described murine B_1 cells, present in spleen and bone marrow, and B_2 cells, found mainly in spleen. B_1 cells require T cell help in their response to antigen while B_2 cells are T cell independent. However, it has recently been demonstrated that T cells can influence the class and subclass of antibody produced by B cells after contact with 'T-independent' antigens (Mongini, Stein and Paul, 1981).

B lymphocyte subpopulations have been characterised by their differential expression of IgM and IgD. Ault and Towle (1981) demonstrated that the small number of sIgG bearing B cells are responsible for the PWM response.

Owing to the difficulties encountered in B cell identification, the characterisation of B lymphocyte subpopulations remains incomplete. Much of the published work relates to murine B cells. However, the recent production of a human B lymphocyte hybridoma antibody may help to clarify the picture. At present, the only specific B lymphocyte markers are considered to be surface (membrane incorporated) IgM and EBV receptors.

(iv) Null Lymphocytes

This section will consist of an introduction to the various methods for isolation of null cell populations followed by a report of cell characterisation and functional studies.

Introduction

A population of human peripheral blood lymphocytes lacking conventional T and B cell markers has been described as "null" lymphoid cells (Kalden et al, 1977; Niandet, Greaves and Horwitz, 1979), non-T, non-B third population cells (Frøland, Wisløff and Michaelsen, 1974), L lymphocytes with membrane labile IgG (Horwitz and Lobo, 1975) and undefined lymphocyte like (UL) cells (Dickler, 1976 b). These terms are not synonymous but the populations overlap and all appear to contain cells with high avidity Fc receptors.

Detection of Null Cell Populations

Third Population Lymphocytes

EA rosette formation is commonly used for the detection of

Fc receptor (FcR) positive lymphocytes (WHO/IARC, 1974). Frøland, Wisløff and Michaelsen (1974) demonstrated that human group O erythrocytes sensitised with Ripley anti CD antiserum were selectively bound by human peripheral blood non-T, non-B lymphocytes. Several EA rosetting systems were compared; Natvig and Frøland (1976) achieved similar results using other anti-rhesus isoantibodies with anti-CD specificity. Pang and Wilson (1978) confirmed the observations of Frøland and co-workers (1974). Using sensitised ox erythrocytes (EA_{OX}) and human erythrocytes coated with anti-CD IgG (EA_{CD}), they showed that both B and null FcR positive cells formed rosettes with EA_{OX} whereas B cells did not form EA_{CD} rosettes. Also, B cells required high concentrations of sensitising antibody for rosette formation whereas null cells formed rosettes at low concentrations of antibody. More recently, it has been suggested that cell populations detected by EA_{CD} and EA_{OX} are identical (Dunne, Spiegelberg and Vaughan, 1981). Other workers have demonstrated binding of Ripley sensitised erythrocytes by cells other than null cells (Shaw, Levy and Lobuglio, 1979; Dobloug et al, 1979; Andersson and Svennevig, 1980).

UL Lymphocytes

UL cells have been shown to have high avidity Fc receptors in that soluble antigen-antibody complexes were bound even when present at very low concentrations (Arbeit, Henkart and Dickler, 1977). This agrees with the observations of Pang and Wilson (1978). In these studies UL cells were defined as lymphocytes bearing cytophilic membrane immunoglobulin.

L Lymphocytes

L lymphocytes are the C3 receptor (C3R) - negative subset of non-T, non-B cells with high avidity, trypsin resistant, cold reactive Fc receptors which comprise about 95% of all null cells. L lymphocyte populations are prepared by the removal of E rosette and C3R positive cells from whole lymphocyte populations (Lobo, Westervelt and Horwitz, 1975; Lobo and Horwitz, 1976; Horwitz, 1977). L cells were found to be divided into two subsets of C3R negative lymphocytes; about 60% of L cells were shown to be Ia positive while approximately 60% reacted with $F(ab')_2$ fragments of an anti-myeloid IgG. A small number (10-15%) of Ia positive, myeloid positive L cells were detected and these showed some morphological features of monocytes (Horwitz, Niaudet, Greaves, Dorling and Deteix, 1978).

Null Cells

Null cell populations contain L lymphocytes and a small population of C3R positive non-T, non-B cells. However the term "null" is often applied to all the populations described here. Niaudet, Greaves and Horwitz (1979) demonstrated a slightly increased percentage of Ia positive cells in null compared with L cell preparations. This was probably due to the presence of Ia positive, $Fc\gamma R$ positive, C3R positive cells. Null cells are usually obtained by negative selection.

Null Cell Characterisation

Since the majority of bone marrow cells lack surface markers

(Abdou, Alavi and Abdou, 1976; Yang et al, 1978), many investigators believed that null cells were immature cells. This question remains unanswered. It has been shown that some null cells are immature B cells. Chess, Levine, MacDermott and Schlossman (1975) demonstrated sIg on cultured null cells and compared Ig content and Ig secretion of cultured B and T cells with cultured null cells; the values obtained with B and null cells were similar while T cells showed no Ig synthesis and no measureable Ig content. In contrast to the findings of Chess and co-workers (1975), Horwitz and Garrett (1977) concluded that L lymphocytes were not a subset of B cells since L cells did not spontaneously develop sIg in culture and PWM did not induce transformation and Ig production.

Mixed antiglobulin rosetting reactions were developed (Haegart and Coombs, 1976) and Haegart (1979) demonstrated integral surface immunoglobulin on L lymphocytes. Haegart, Hurd and Coombs (1978) showed that less than 1% of all peripheral blood lymphocytes were null cells and less than 5% had B and T cell markers. This finding strengthened the argument that null cells are a population of B cells (Haegart and Coombs, 1979; Haegart, 1980).

Horwitz and co-workers (1978), using direct immunofluorescence and mouse erythrocyte rosette formation found only 8% positive cells in the L population and supposed these to be contaminating B cells. The same workers (Horwitz et al, 1978) defined 11% of the L population as "contaminating T cells" since they reacted with anti human T lymphocyte antiserum and with sheep erythrocytes. The L cell population was esterase and peroxidase negative. Niaudet, Greaves and Horwitz (1979) and Horwitz and colleagues (1978) showed

high reactivity of null populations with anti Ia and anti myeloid sera. More recently, Kay and Horwitz (1980), using hybridoma antibodies, showed reactivity of null cells with OKM1 but confirmed their non-phagocytic, α-naphthyl esterase negative properties.

Functional Characterisation of Null Cells

Lymphocyte Stimulation

As previously stated, Horwitz and Garrett (1977) showed that PWM did not induce L lymphocyte transformation. Protein A is also non mitogenic to L lymphocytes (Sakane and Green, 1978). Horwitz and Garrett (1977) found L lymphocytes to be ineffective stimulators of autologous and allogeneic mixed lymphocyte cultures depleted of monocytes and L cells could not replace monocytes in helping T cells respond to Con A and PWM; however, the addition of L lymphocytes to B cell depleted T lymphocyte preparations containing monocytes greatly enhanced the response of T lymphocytes to mitogens and soluble antigens. The observed enhancement of blastogenesis was studied in detail (Carvalho, Davis and Horwitz, 1980). Peripheral blood mononuclear cells from individuals sensitised to Keyhole limpet haemocyanin (KLH) and healthy controls were stimulated *in vitro* with KLH and KLH-anti KLH immune complexes. Only sensitised individuals responded and immune complexes produced a higher response than antigen alone. Enhancement was greatest after 3-5 days in culture and was dependent on an intact Fc region since immune complexes prepared with the $F(ab')_2$ portion of antibody and Fc receptor negative lymphocyte populations did not produce enhancement. T cell-L cell combinations were studied using mitomycin treatment to block cell division. Thus T but not L cells participated in the

blastogenic response and L cells amplified T cell reactivity. This work led to the assumption that L cells are accessory cells derived from myeloid stem cells (Carvalho, Davis and Horwitz, 1980; Horwitz and Carvalho, 1980).

Cell Mediated Cytotoxicity

The cytotoxic B cell was described by MacLennan (1972) and defined as a non-T lymphocyte. Wisløff, Frøland and Michaelsen (1973) ascribed cytotoxic potential to their third population lymphocytes. Other workers confirmed that antibody dependent cellular cytotoxicity (ADCC) was independent of B cells and required lymphocytes with high avidity FcRs (Wisløff and Frøland, 1973; Revillard et al, 1975; Papamichail and Temple, 1975; Perlmann et al 1975; Brier, Chess and Schlossman, 1975; Perlmann, Perlmann, Pape and Hallden, 1976; Horwitz and Garrett, 1977; Ziegler et al 1977; Pape, Troye and Perlmann, 1977). The effector cells have been termed killer (K) cells. ADCC activity has been found in many Fc receptor positive cell populations; thus T_γ lymphocytes (described in the previous section), B lymphocytes (Brier, Chess and Schlossman, 1975), monocytes (Papamichail and Temple, 1975; Hammarstrøm, 1979; Norris et al, 1979), polymorphonuclear leucocytes (Shaw, Levy and Lobuglio 1979) and null lymphocytes all mediate ADCC.

The K effector cell has been described as a villous cell (Perlmann et al, 1975) which bears a receptor for C3 (Perlmann et al, 1976). Others workers find no requirement for C3 receptors (Horwitz and Garrett, 1977; Johnsen and Madsen, 1979 b). Using Helix pomatia lectin (HP) affinity column chromatography, Hellstrøm

and colleagues (1976) found enhanced K cell activity in the HP negative population over the HP positive population. The HP positive lymphocytes were also sIg positive but this was probably externally absorbed IgG and thus this fraction is probably a T_γ cell fraction. K cells were shown to be Ia negative (Nelson, Sachs and Dickler, 1977).

Null cells have also been implicated in spontaneous cell mediated cytotoxicity (SCMC). In murine systems these have been extensively studied. Kiessling and colleagues (1976) showed that murine NK cells lacked C3 receptors but K cells formed EAC rosettes, that heat aggregated IgG inhibited ADCC but not SCMC and that NK cell activity was sensitive to trypsin treatment while K cell activity was resistant to trypsin. A cell surface antigen was identified which was selectively expressed by natural killer (NK) cells (Ly 5); these cells did not express Thy-1, Ly 2 or Ig surface markers (Glimcher, Shen and Cantor, 1977). Nude mice (congenitally athymic) have high NK activity, thus indicating a non-T nature for these cells.

In man, NK activity hws been demonstrated in null Fc receptor positive cell populations, although the Fc receptor does not seem to be necessary for activity (Muchmore, Decker and Blaese, 1977). Using HP affinity chromatography, NK activity was found in HP positive and HP negative populations; the SCMC activity of the HP negative cells was always higher than the unfractionated cells (Pape, Troye and Perlmann, 1979). Spontaneous cytotoxicity by lymphocytes from healthy donors shows considerable variability both between donors and in the same donor over several months (Santoli et al, 1978).

Comparisons of ADCC and SCMC are often made since isolated null cells contain both activities. Both human K and NK cells are inhibited by protein A (Kay et al, 1977) and null effector cells have been shown to lack 'Ia-like' antigens, (Ozer et al, 1979). The separation of K and NK cells has proved difficult. Kalden and co-workers (1977) showed that sIg negative lymphocytes depleted of high avidity FcR bearing cells were still capable of killing and SCMC was enhanced. Recently, Neville (1980) successfully separated K and NK lymphocytes using different glutaraldehyde treated cell lines and NK cells were shown to have less avid Fc receptors.

The null lymphocyte subset is thus very diverse containing cytotoxic cells and accessory cells. A suppressor function has also been postulated (Frøland and Abrahamsen, 1979). Null cell populations are usually obtained by negative selection but the recent production of a hybridoma antibody may facilitate future separation and characterisation (Beckman et al, 1980).

SECTION II

THE IMMUNOPATHOGENIC ROLE OF LYMPHOCYTES IN RHEUMATIC DISEASE

Rheumatoid arthritis (RA) is a connective tissue disease of unknown aetiology which manifests itself both locally and systemically but is characterised chiefly by an inflammatory erosive polyarthritis.

In this section, the pathological changes occurring in RA will be outlined and a possible aetiology briefly discussed. The majority of the section will be restricted to an account of lymphocyte populations in RA with respect to their surface markers and functions and differences between healthy and rheumatoid lymphocytes will be reported. Lymphocyte abnormalities in other rheumatic diseases relevant to work in this thesis will be summarised.

RHEUMATOID ARTHRITIS (RA)

(i) General Introduction

Anatomy, Pathology and Pathogenesis

Glynn (1972) described the three outstanding anatomical features of RA to be "inflammation with progressive deformity of joints, subcutaneous nodules, ... and vascular lesions ..."

A normal diarthrodial synovial joint consists of two bone ends capped with articular cartilage and joined by a fibrous capsule which is contiguous with the periosteum of the bones. The synovial

membrane or synovium lines the capsule and is composed of a thin layer of cells (Swinson and Swinburn, 1980). The lining layer of the synovium, the intima, is 1-3 cells in thickness and rests on a very vascular subintimal tissue which may be adipose, fibrous or areolar. Synovial cells or synoviocytes are responsible for the secretion of hyaluronic acid into the synovial fluid, the removal of solid particles from the joint cavity and the activation of fibrinogen which is thought to prevent the accumulation of fibrin in the joint (Fell, 1978). The intimal cells have been shown to be of two types, A cells which are phagocytic and resemble macrophages and B cells which are characterised by abundant, well-developed, rough-surfaced endoplasmic reticulum and are fibroblastic in appearance. The A cells are the predominant cell type. It is uncertain which types are responsible for specific functions (Zvaifler, 1973; Fell, 1978).

In health, synovial fluid is thick, clear and viscous and contains hyaluronic acid. Normal synovial fluid does not clot; fibrinogen is absent in normal fluid but all the components of the fibrinolytic system necessary to produce free plasmin are present. Plasmin is the enzyme normally involved in fibrin removal. Normal joints contain small volumes (< 2 ml) of synovial fluid (Zvaifler, 1973; Swinson and Swinburn, 1980).

Early RA is characterised by swollen joints and proliferative synovitis. Within the joint, excess connective tissue or "pannus" is produced by the proliferation of synovial lining cells with extensive vascularisation and infiltration of lymphocytes, plasma cells, polymorphonuclear leucocytes and macrophages (Harvey, 1978);

the lining cells increase to a depth of 6-10 cells (Zvaifler, 1973) and the pannus protrudes into the joint space. Harris (1976) suggested that irreversible joint destruction may begin with the degradation of articular cartilage which is mediated by the encroaching pannus. Chayen and Bitensky (1971) have demonstrated a high lysosome content in rheumatoid synovial lining cells and the presence of lysosomal enzymes in rheumatoid joints; these may contribute to the inflammation.

The rheumatoid synovial membrane has been studied using electron microscopy. Kobayashi and Ziff (1973) identified perivascular accumulations rich in lymphocytes or plasma cells or both cell types. Gaucher and colleagues (1976) characterised cells of varying sizes connected by intercellular bridges and thus rheumatoid synovial membrane presented a villous surface in comparison to the smooth flat surface of normal synovial membrane. Hyperplasia and hypertrophy is a characteristic feature of rheumatoid joints.

Rheumatoid synovial fluid varies considerably from normal fluid. Perhaps the most striking differences are the total volume of the fluid (as much as 200-400 ml in active RA) and the ability of rheumatoid synovial fluid to clot (Zvaifler, 1973). Rheumatoid synovial fluid protein and lipid levels are grossly elevated while the haemolytic complement activity is significantly depressed; in contrast, serum complement levels in rheumatoid patients are either normal or slightly increased.

It is obvious that cells infiltrate the joint from the surrounding tissues or blood vessels. Zvaifler (1973) has described

the predominant cell in rheumatoid synovial fluids as a mature polymorphonuclear granulocyte accounting for 75-90% of all nucleated cells. The remaining cells consist of 5-10% lymphocytes with some monocytes, macrophages and synovial lining cells; all may be involved in the pathogenesis of RA. Direct evidence for the detrimental effects of lymphocytes in RA was provided by Paulus and co-workers (1977). Thoracic duct drainage produced a clinical improvement in rheumatoid patients and reinfusion of the cells induced exacerbation of disease activity.

Humoral Immunity

The presence of antibodies against the Fc region of autologous IgG (Rheumatoid factors; RFs) in the serum of approximately 80% of patients with RA has been known for many years (Waaler, 1940; Rose, Ragan, Pearce and Lipman, 1948) and has been extensively reviewed (Zvaifler, 1973; Johnson and Faulk, 1976; Ziff, 1980). Classical IgM rheumatoid factor (IgMRF) led to the definition of seropositive or seronegative RA. No correlation was found between IgMRF and raised immunoglobulin levels in rheumatoid patients (Barden, Mullinax and Waller, 1967). More recently IgG rheumatoid factor has been demonstrated in both seropositive and seronegative rheumatoid patients but not in other seronegative rheumatic diseases (Allen et al, 1981). High levels are associated with rheumatoid vasculitis. IgA rheumatoid factors have also been described (Johnson and Faulk, 1976; March et al, 1981).

Rheumatoid factors consequently lead to the production of circulating antigen-antibody complexes and thus RA is often termed

an immune complex disease. Complexes can interfere with Fc receptor mediated functions and, once deposited in the tissues, may have pathological effects. Glomerulonephritis may be immune complex induced (Wiggins and Cochrane, 1981) although this is rare in RA.

The rheumatoid synovial membrane is known to synthesise immunoglobulin of mainly IgG class (Smiley et al, 1968); IgG-IgG complexes can be synthesised intracellularly and secreted. Approximately 50% of the plasma cells are synthesising IgG (Natvig and Munthe, 1975). Immune complexes containing IgM, IgG and complement have been detected in synovial fluids (Zvaifler, 1973). Britton and Schur (1971) described intracytoplasmic inclusions of immunoglobulins and complement in synovial fluid leucocytes and suggested that immune complexes form in the synovial fluid, fix complement and through activation of C3 are phagocytosed by synovial fluid leucocytes. This may provide an explanation for low complement levels in the joint.

Other autoantibodies have also been documented. Anti nuclear antibodies (ANAs) have been described and many reviews are available (Holborow, 1979). These antibodies are mainly found in patients with systemic lupus erythematosus (SLE) although they are sometimes found in rheumatoid patients. Grennan, Sloane, Behan and Dick (1977) showed that rheumatoid patients with ANAs to double stranded DNA are more likely to have severe disease and extra-articular complications. Anti collagen antibodies have been detected in both rheumatoid serum and rheumatoid synovial fluid and collagenase activity has been shown to be present in rheumatoid synovial membranes and synovial fluid (Steffen, 1980).

Cell Mediated Immunity

Cytotoxicity measurements, antigen and mitogen responsiveness and suppressor activity will be discussed later in Section II with respect to discrete lymphocyte populations.

The human leucocyte migration inhibition test (LMT) has been used widely to assay cellular responses to antigen (Bendixen et al, 1976). Patients with RA respond abnormally to autologous IgG (Weisbart, Bluestone and Goldberg, 1975). The abnormal response is more marked if the IgG is altered in some way, for example by complexing to antigen or by heat aggregation (Eibl and Sitko, 1975; Hall, 1978). Circular dichroism studies have been used to show that rheumatoid IgG has an altered structure (Johnson, Watkins, Scopes and Tracey, 1974) and it was postulated that this alteration in structure may cause autoantibody production resulting in immune complex formation (Johnson, Watkins and Holborow, 1975). This remains unproven.

Aetiology

The search for a primary antigen in RA has so far proved fruitless. It was originally thought that rheumatoid factor was the cause of RA but the discovery of high titres in other diseases dispelled the assumption (Zvaifler, 1973; Johnson and Faulk, 1976; Carson et al, 1978). Using the LMT, some workers have demonstrated a specific response by rheumatoid pateints to synovial eluates (Bacon et al, 1973; Robinson and Muirden, 1980). Other investigators, using very large numbers of patients, have shown this effect to be non-specific (Morgan et al, 1980).

An infectious agent has been implicated as the instigator of RA since many immunopathological features resembling those of connective tissue diseases are associated with infectious diseases. It is known that arthritis complicates many bacterial and virus infections, such as Rubella, although it is nearly always short-lived and non-erosive. Also, a wide variety of autoantibodies are produced in some infectious diseases such as subacute bacterial endocarditis (SBE) or leprosy; anti-IgG can neutralise infectious complexes of antigen bound to viruses such as Herpes simplex virus (HSV). Thirdly, virus infection has been implicated in some degenerative diseases of the central nervous system (i.e. 'slow virus' infections) and influenza virus has been shown to precede Goodpasture's syndrome, an antibody induced glomerulonephritis in which antibodies to glomerular basement membrane are produced; there is commonly an immune complex nephritis in infections including SBE and leprosy. This subject is reviewed by Denman (1975) and Denman and colleagues (1977); these authors favour a viral aetiology for RA but this is not proven.

Following many viral infections, viral antigens are expressed on the surface of infected cells and the viral genome may become integrated into host DNA, thus persisting in the host cell. Norval and Smith (1979) found no evidence of viral genomes in the DNA and RNA of synovial fibroblasts, synovial membranes and peripheral blood lymphocytes of patients with RA and other joint conditions. Appleford and Denman (1979 a) isolated lymphocytes from the peripheral blood and synovial fluid of rheumatoid patients and showed that only peripheral blood lymphocytes supported the growth of Herpes simplex virus (HSV). The same workers showed that T lymphocytes isolated

from synovial fluid could support the growth of HSV but the re-addition of non-T lymphocytes again caused non-permissiveness. However, if synovial fluid non-T lymphocytes were added to autologous peripheral blood T cells, no such effect was noted and the virus grew normally. Removal of glass adherent cells from non-T synovial fluid lymphocytes did not remove inhibition. The authors suggest that synovial non-T cells may be producing anti viral factors (Appleford and Denman, 1979 b).

Other investigators suggesting a viral aetiology for RA described the presence of a serum antibody in rheumatoid patients which is reactive with a soluble nuclear antigen extracted from human lymphoblastoid lines infected with Epstein Barr Virus (EBV) but not those infected with other Herpes type viruses. This RA associated nuclear antigen (RANA) appears to be distinct from Epstein Barr nuclear antigen or EBNA (Alspaugh et al, 1978). A high frequency of anti-RANA antibody was shown to occur in both seropositive and seronegative RA. Normal controls and patients with other arthritides had low levels of the antibody (Ng et al, 1980). Catalano and co-workers (1980) studied healthy individuals and showed that those having antibodies to viral capsid antigen (VCA), which is expressed during EBV infection, also had anti-RANA antibodies while anti-VCA negative individuals were anti-RANA negative. They concluded, therefore, that anti-RANA antibodies are produced only after EBV infection. Ferrell and co-workers (1981) confirmed the high incidence of anti-RANA antibodies in rheumatoid patients and the distinction from anti-EBNA. Other EBV-associated antibodies were detected; thus rheumatoid patients had elevated levels of anti-VCA and antibody to early antigen, another EBV related antigen, was present.

Anti-Rubella antibody secreting cells have been demonstrated in the peripheral blood of patients with RA (H. Chattopadhyay et al, 1979 a). Later work showed that although the antibody response to viral antigens was increased, rheumatoid patients were hyporesponsive to those antigens when tested in the LMT (H. Chattopadhyay, C. Chattopadhyay and Natvig, 1979 b).

Genetic Factors

Rheumatoid arthritis is a disease of relatively recent origin (Bywaters, 1975). It has been suggested that environmental factors may effect genetically predisposed individuals to develop the disease.

A D locus related antigen DRw4 was detected in 70% of caucasian rheumatoid patients in comparison with 28% of normal controls (Stastny, 1978). Panayi and Wooley (1977) screened 116 sera from multiparous women made specific for HLA-D antigens. One serum gave results analogous to those of Stastny (1978). In contrast the antigens HLA-DRw7 and HLA-DRw8 were increased in children with JCA while HLA-DRw4 occurred significantly less frequently than in normal controls (Stastny and Fink, 1979).

The antigen DRw1 appears to map RA in Indian patients (Nichol and Woodrow, 1981).

It thus appears that much is known of the pathological changes occurring in RA but little is known of the aetiology; the causative agent may be a combination of two or more of the factors described above (Vaughan, 1979).

(ii) Lymphocyte Subpopulations in Rheumatoid Arthritis

Rheumatoid lymphocyte subpopulations have been enumerated using surface markers, responses to mitogen or antigen and functional characteristics. The lymphocyte populations present in peripheral blood, synovial fluid and synovial tissue will be presented separately. Each group will be divided into T and non-T cells to avoid the confusion described in Section I concerning sIg positive and null Fc receptor bearing cells. Antibody-dependent and spontaneous cell mediated cytotoxicity will be included in the non-T section.

Peripheral Blood Lymphocyte Subpopulations in Rheumatoid Arthritis

(a) T Lymphocytes

Cell Numbers

Most workers agree that peripheral blood T cells in RA are present in normal numbers (Micheli and Bron, 1974; Sheldon, Papamichail and Holborow, 1974; Winchester et al, 1974; Brenner, Scheinberg and Cathcart, 1975; Scherak et al, 1976; Horwitz and Juul-Neilsen, 1977; Meijer et al, 1980 a; Slavin and Strober, 1981) although Burmester and colleagues (1978) have reported reduced numbers while other workers found increased numbers (Frøland, Natvig and Husby, 1973; Frøland, Natvig and Wisløff, 1975). Roux and co-workers (1977) showed that rheumatoid patients had either normal or decreased T cell numbers. All patients with low T cell numbers in this study were lymphopaenic but this was not correlated with disease activity or drug therapy.

Normal levels of the T_γ subset have been reported in RA

(Meijer et al, 1980 a; Pfreundschuh et al, 1980; Dobloug et al, 1981) although Dobloug and associates (1981) noted reduced T_γ in JCA. Levels of T_μ cells have been reported as normal (Doubloug et al, 1981), increased (Pfreundschuh et al, 1980) or decreased percentages (Meijer et al, 1980 a). The latter authors found some correlation between low T_μ cell numbers and disease activity.

Increased Ia positive T lymphocytes have been reported in rheumatoid patients and these were present in both the T_γ and non-T_γ subsets of T cells. It was noted that these Ia positive T cells were small lymphocytes; in vitro stimulated Ia positive cells are blasts (Yu et al, 1980).

Mitogen Responsiveness

The ability of rheumatoid peripheral blood lymphocytes (PBL) to respond to plant mitogens has been widely studied and diverse results have been obtained. Lockshin and co-workers (1975) described a decreased response of rheumatoid PBL to PHA, Con A and PWM in comparison with healthy controls. They were unable to correlate this lack of response with E rosette positive lymphocytes. In the control group, normal responses could be obtained with as few as 10% T cells; this low proportion of T cells is not seen in RA. Burmester and associates (1978) found a decreased response of rheumatoid PBL to PHA and Con A but this decrease was not significantly different from control values. The responses to PPD and PWM were also similar to those of control lymphocytes. Froebel and colleagues (1979) showed reduced responses to PHA which were just significant at low doses. The responses to Con A and PWM were also slightly

diminished but these were not significantly different from control values. Other workers have also shown that rheumatoid peripheral blood lymphocyte responses to phytomitogens are not significantly different from those of healthy individuals (Panayi, 1973; Sheldon, Papamichail and Holborow, 1974; Meijer et al, 1980 b).

Slavin and Strober (1981) found a decreased PHA responsiveness of PBL in active RA but isolated rheumatoid T lymphocytes showed no such decrease in PHA responsiveness. The addition of rheumatoid sera from patients with active disease inhibited the PHA transfomation of both normal PBL and rheumatoid T lymphocytes. A defect in Con-A induced suppression was noted by Sany and colleagues (1979) while Dobloug and associates (1981) reported a slightly reduced suppressor activity in RA but this was not significantly different from control values. Normal suppressor activity was seen in JCA patients. Other workers have demonstrated a reduced Con-A suppressor T cell function in early active rheumatoid patients (< 3 months). Inactive and late active (> 6 months) rheumatoid patients showed no suppressor cell abnormalities. The early active patients, when tested 11-23 months later, showed normal suppressor activity. An anti-suppressor cell antibody was detected in the plasma of early active patients (Abdou et al, 1981). Meijer and co-workers (1980 a) found no antibodies to T_γ in rheumatoid sera and anti-T_λ was present only in active disease. These studies demonstrate the necessity of well-defined patient groups.

Mitogen-induced Cellular Cytotoxicity

Fewer studies have been performed on the ability of PHA to

induce cytotoxicity. Corrigall and Panayi (1978) showed no significant differences in PHA-induced cytotoxicity in rheumatoid patients when compared with patients with other inflammatory joint diseases. Rosenberg and Currey (1979) found no significant differences in the PHA-induced cytotoxicity of rheumatoid and healthy PBL.

(b) Non-T Lymphocytes

Cell Numbers

sIg positive lymphocytes

There is one report of increased sIg positive cells in rheumatoid peripheral blood (Papamichail, Brown and Holborow, 1971) but the majority of workers agree that these cells are present in normal numbers in PBL (Mellbye et al, 1972; Frøland, Natvig and Husby, 1973; Micheli and Bron, 1974; Sheldon, Papamichail and Holborow, 1974; Brenner, Scheinberg and Cathcart, 1975; Frøland, Natvig and Wisløff, 1975; Horwitz and Juul-Nielsen, 1977; Burmester et al, 1978; Meijer et al, 1980 a; Slavin and Strober, 1981). Froebel and colleagues (1979) reported significantly raised numbers of sIg positive cells in rheumatoid patients in remission.

C3R Positive Lymphocytes

Mellbye and colleagues (1972) found significantly reduced numbers of C3R positive PBL in adult RA whereas patients with JCA had normal numbers. Other workers described normal levels of C3R positive cells in RA (Brenner, Scheinberg and Cathcart, 1975; Burmester et al, 1978).

Fc Receptor Bearing Lymphocytes

The "rheumatoid rosette" was described by Bach, Delrieu and Delbarre (1970). Peripheral blood lymphocytes from both seropositive and seronegative patients bound antibody sensitised O rhesus negative erythrocytes. Increased numbers were detected in RA, particularly in early disease. No correlation with IgM rheumatoid factor was found. Rheumatoid rosettes were also elevated in patients with gout. Sany, Clot, Massip, Charmasson and Serre (1975) showed that rheumatoid rosette forming lymphocytes were neither T nor B cells since E rosette depletion and nylon filtration increased the proportion of rosette forming cells detected. Other workers have confirmed increased EA rosette formation in RA (Scherak et al, 1976; Sharpin and Wilson, 1977 a). Sharpin and Wilson (1977 b) used whole serum for erythrocyte sensitisation but demonstrated that the EA rosettes formed were due to $Fc_{\gamma}Rs$ only. Durance, Micheli and Fallet (1974) were unable to find a significant difference in rheumatoid rosettes between rheumatoid and gout patients and controls. Two other groups of workers also demonstrated normal EA rosette numbers in RA (Burmester et al, 1978; Froebel et al, 1979).

Wooley and Panayi (1978) showed increased binding of radiolabelled heat aggregated IgG by rheumatoid cells compared with controls. The authors concluded that the observed increase may be due to increased numbers of FcRs per cell or increased avidity of FcR on rheumatoid cells.

Third population cells, detected by Ripley sensitised erythrocytes, have been found in normal numbers in rheumatoid peripheral

blood (Frøland, Natvig and Wisløff, 1975; Dobloug et al, 1981). This null population appears to have membrane characteristics which differ from controls since lower numbers are detected following nylon fibre filtration (Frøland, Natvig and Wisløff, 1975). L cells are present in normal percentages but their absolute numbers are depressed (Horwitz and Juul-Nielsen, 1977).

Antibody Dependent Cellular Cytotoxicity (ADCC)

Several groups of workers have found ADCC activity to be unaltered in RA. A variety of targets have been used including chicken erythrocytes (Frøland, Natvig and Wisløff, 1975; Cooke, Hay and Perumal, 1979; Rosenberg and Currey, 1979), Chang liver cells (Panayi and Corrigall, 1977; Penschow and Mackay, 1980) and a human melanoma cell line, IGR_3 (Burmester et al, 1978).

McGill and Twinn (1977), using chicken erythrocytes as targets, related disease activity to cytotoxic activity; active seropositive rheumatoids had reduced ADCC activity while the seronegative patients showed normal activity. Froebel and associates (1979) showed reduced peripheral blood ADCC against Chang liver cells in active RA. Patients with early RA ($<$ 1 yr) and chronic rheumatoid patients in remission had normal activity. EA rosette formation correlated with ADCC activity.

Rheumatoid rosette forming cells were found to be active in ADCC (Sany et al, 1976). Removal of rheumatoid rosette forming cells abolished ADCC and removed EA rosette forming cells. Removal of EA rosette forming cells removed rheumatoid rosette forming cells

and also abolished ADCC. In contrast to other reports ADCC activity against chicken erythrocytes was found to be increased in rheumatoid patients.

Panayi (1976) described the presence in 31% of rheumatoid sera of an IgG antibody which induces healthy PBL to kill Chang target cells. Anti-Chang activity was detected in only 4% of control subjects and similar results were obtained with other connective tissue diseases. In contrast, Cooke, Hay and Perumal (1979), using smaller patient groups and chicken erythrocytes as target cells, found no significant difference in normal ADCC activity when rheumatoid sera were included in the assay but the activity was significantly inhibited by sera from patients with other connective tissue diseases in comparison to normal human serum.

Spontaneous Cell Mediated Cytotoxicity (SCMC)

Using Chang cell targets, Penschow and Mackay (1980) found normal SCMC in the PBL of RA patients. Highton and Panayi (1980) examined the spontaneous cytotoxicity of RA and normal PBL against four lymphoblastoid cell lines including one T cell line and 3 B cell lines. Two of the B cell lines were positive for EBV genome. No significant differences in killing were seen in the PBL of rheumatoid or normal individuals; there was no preferential recognition of EBV-associated antigens. High SCMC was mediated against the T cell line.

Burmester and co-workers (1978), using a human melanoma cell line, described decreased SCMC by rheumatoid peripheral blood at

high lymphocyte/target ratios (100:1); effector/target ratios below this showed no significant decrease.

Synovial Fluid Lymphocyte Subpopulations in Rheumatoid Arthritis

Synovial fluid cells are usually compared with peripheral blood lymphocytes of rheumatoids or controls.

(a) T Lymphocytes

Cell Numbers

Rheumatoid synovial fluid lymphocytes (SFL) are predominantly T cells and are present in significantly higher numbers than in the peripheral blood of either RA or normal individuals. (Frøland, Natvig and Husby, 1973; Vernon-Roberts, Currey and Perrin, 1974; Winchester et al, 1974; Sheldon, Papamichail and Holborow, 1974; Frøland, Wisløff and Michaelsen, 1975; Brenner, Scheinberg and Cathcart, 1975; Van de Putte et al, 1976).

One group of workers reported similar numbers of T cells in PBL and SFL (Burmester et al, 1978) and decreased numbers of T cells were found in JCA (Brenner, Scheinberg and Cathcart, 1975). High synovial fluid T cell numbers have been described in a wide variety of other arthritides (Van de Putte et al, 1976).

Mitogen Responsiveness

Synovial fluid lymphocytes from patients with RA were significantly less reactive than autologous PBL in their response to

PHA, Con A and PWM (Stratton and Peter, 1978). It is generally accepted that synovial fluid lymphocytes respond poorly to PHA (Panayi, 1973 ; Sheldon, Papamichail and Holborow, 1974; Abrahamsen, Frøland and Natvig, 1978; Burmester et al 1978; Corrigall, Panayi and Laurent, 1979). The poor response is not due to blocking agents adsorbed on the lymphocyte surface since incubation at 37°C or enzyme treatment does not enhance lymphocyte responsiveness (Corrigall, Panayi and Laurent, 1979). Decreased PHA responses have also been reported in synovial fluid of JCA patients (Abrahamsen, Frøland and Natvig, 1978).

Synovial fluid lymphocytes have a high background DNA synthesis before mitogen stimulation and it has been postulated that SFL are a pre-committed "activated" population.

Con A responses of SFL have been described as decreased (Burmester et al, 1978) or normal (Corrigall, Panayi and Laurent, 1979). Similarly, PWM responses were reported as reduced (Abrahamsen, Frøland and Natvig, 1978) or normal (Burmester et al, 1978; Corrigall, Panayi and Laurent, 1979).

Responses to PPD and Candida albicans antigen have been described in RA and JCA. Rheumatoid synovial fluid lymphocytes stimulated with PPD showed significantly higher responses than autologous PBL whereas responses of JCA patients were normal (Abrahamsen, Frøland and Natvig, 1978). Other workers found no significant differences in the responses of SFL or PBL from rheumatoid patients to PPD (Burmester et al, 1978). Both RA and JCA patients showed normal responses to Candida antigen when PBL and SFL responses were compared (Abrahamsen, Frøland and Natvig, 1978).

Mitogen-induced Cellular Cytotoxicity

No significant differences in mitogen-induced cytotoxicity were observed in SFL of RA patients when compared to PBL of RA and the SFL and PBL of patients with other inflammatory joint diseases (Corrigall and Panayi, 1978).

(b) Non-T Lymphocytes

Cell Numbers

sIg positive cells

An early study by Mellbye and associates (1972) reported significantly higher sIg positive cells in rheumatoid joint fluid when compared to peripheral blood but this was based on raised sIg bearing cells which are probably mainly L cells. Some workers have reported no significant differences in numbers when compared to PBL (Vernon-Roberts, Currey and Perrin, 1974; Brenner, Scheinberg and Cathcart, 1975; Burmester et al, 1978) while other groups found decreased numbers of sIg positive cells; in some cases this depression is very marked (Frøland, Natvig and Husbuy, 1973; Sheldon, Papamichail and Holborow, 1974; Winchester et al, 1974; Frøland, Natvig and Wisløff, 1975; Van de Putte et al, 1976). Vernon-Roberts, Currey and Perrin (1974) detected significantly raised IgA positive cells in rheumatoid synovial fluid.

C3 positive Lymphocytes

Synovial fluid C3 positive lymphocytes have been described as normal (Mellbye et al, 1972) or reduced (Brenner, Scheinberg and Cathcart, 1975; Burmester et al, 1978).

Fc-receptor Positive Lymphocytes

EA rosettes were found in similar numbers in PBL and SFL of rheumatoid patients (Burmester et al, 1978). Third population cells are present in rheumatoid synovial fluid (Frøland, Natvig and Wisløff, 1975) and also in the fluid of JCA patients; the levels are not significantly different from those of peripheral blood (Abrahamsen, 1981).

Null cells in synovial fluid have been calculated by negative inference (i.e. 100 - (%T + %B)). Brenner, Scheinberg and Cathcart (1975) found high levels of null cells in synovial fluid from JCA patients. Rheumatoid fluids showed very low or normal to high levels of null cells; however this bimodal distribution yielded a normal mean value. Vernon-Roberts, Currey and Perrin (1974) found significantly reduced null cell values in rheumatoid synovial fluid. Winchester and co-workers (1974) demonstrated high levels of null cells in rheumatoid synovial fluid detected by binding of aggregated IgG.

Antibody-Dependent Cellular Cytotoxicity

Several groups of workers have detected reduced ADCC in synovial fluid in comparison with peripheral blood and a number of target cells were employed, including chicken erythrocytes (McGill and Twinn, 1977; Cooke, Hay and Perumal, 1979), Chang cells (Corrigall and Panayi, 1978) and human melanoma cells (Burmester et al, 1978). Abrahamsen (1981) using chicken erythrocytes as targets, found no significant differences between peripheral blood and synovial fluid

lymphocytes of patients with RA or JCA. Effector cells were incubated overnight prior to assay.

Corrigall and Panayi (1978), using Chang cell targets, found no increase in cytotoxicity with overnight incubation at 37°C or with neuraminidase or trypsin treatment and demonstrated reduced ADCC in the synovial fluid of other inflammatory joint diseases; the effect is not confined to RA. Panayi (1976) demonstrated anti-Chang activity in some rheumatoid synovial fluids. Cooke, Hay and Perumal (1979) using chicken erythrocytes, described inhibition of normal cytotoxicity by rheumatoid synovial fluid and fluids from patients with other connective tissue diseases. Comparisons were made with normal human serum.

Spontaneous Cell Mediated Cytotoxicity

Most workers found spontaneous cytotoxicity to be raised in RA synovial fluid in comparison to RA and normal peripheral blood (Panayi and Corrigall, 1977; Burmester et al, 1978).

Synovial Tissue Lymphocyte Subpopulations in Rheumatoid Arthritis

Synovial tissue lymphocytes are usually prepared as follows. Synovial tissue is minced, treated with enzymes (collagenase; DNase) and the cell suspension eluted. Lymphocyte populations are usually compared with peripheral blood normal or rheumatoid lymphocytes.

(a) T Lymphocytes

Cell Numbers

Using immunofluorescent staining of synovial tissue sections,

Bankhurst, Husby and Williams (1976) concluded that 75-90% of lymphocytes in rheumatoid tissue are T cells. The same workers eluted cells and this suspension contained 20-30% lymphocytes which were predominantly T cells (78-85%). High T cell numbers were also reported by Abrahamsen and co-workers (1975). Wangel and Klockars (1977) found significantly lower T cell numbers in synovial tissue than in rheumatoid synovial fluid and peripheral blood. However, following trypsin treatment and overnight culture, the E positive cells increased to values similar to those of SFL and PBL. Similarly treated peripheral blood lymphocytes showed no such increase. It was suggested that many eluted synovial cells were T cells with blocked receptors.

Mitogen Responsiveness

Synovial tissue lymphocytes have been reported to show low responses to PHA, Con A and PWM in comparison with PBL, (Abrahamsen, Frøland, Natvig and Pahle, 1976; 1977 a; Meijer et al, 1980 b). Meijer and colleagues (1980 b) also found low respones to Varidase (Streptokinase/Streptodornase) and PPD while Abrahamsen, Frøland, Natvig and Pahle (1976) demonstrated negligible responses to Candida antigen but the response to PPD was remarkably high, showing stimulation indices comparable to those of PHA stimulated synovial tissue lymphocytes.

C. Chattopadhyay and co-workers (1979 a) demonstrated a lack of suppressor cell activity in rheumatoid synovial lymphocytes. Synovial tissue lymphocytes produce high levels of immunoglobulin (Ig) which, following stimulation with PWM, shows a small increase.

In contrast, normal PBL show a significant increase in Ig production when stimulated. At high T/B cell ratios, suppression of Ig production occurs. Synovial tissue T cells were able to help allogeneic normal B cells to respond to PWM but were unable to curtail Ig production at high T/B cell ratios. Further work demonstrated the lack of a con A-activated suppressor activity (C. Chattopadhyay et al, 1979 b).

Non-T Lymphocytes

Cell Numbers

sIg positive Lymphocytes

Wangel and Klockars (1977) detected low numbers of sIg positive synovial tissue lymphocytes. Similar results were obtained by other workers (Abrahamsen, Frøland, Natvig and Pahle, 1976; Meijer et al, 1980 b).

Fc receptor positive Lymphocytes

Third population cells have been detected in low numbers in rheumatoid synovial tissue (Abrahamsen, Frøland, Natvig and Pahle, 1976). Wangel and Klockars (1977) found no significant differences in the FcR positive cells of RA peripheral blood, synovial fluid or synovial tissue detected by binding of heat aggregated IgG.

Antibody Dependent Cellular Cytotoxicity

Both RA and JCA synovial tissue lymphocytes effect ADCC but activity is lower than that of normal PBL. To determine whether the elution procedure was responsible for this reduction, normal PBL

were similarly treated; no change in ADCC was noted. Nylon column filtration resulted in a percentage increase in lymphocytes and decrease in third population cells but cytotoxic activity either increased, decreased or remained the same. Depletion of third population cells gave similarly conflicting activities of ADCC (Abrahamsen, Frøland, Natvig and Pahle, 1977 b).

(iii) Caveat

The analysis of lymphocyte subpopulations and their relative functions in disease is beset by problems. Rheumatoid arthritis is suffered by individuals of all ages and thus age related changes may be mistaken for disease effects. Also, drug treatment may radically alter cell numbers or functions. A brief account of some of these is given below.

Ageing

It is known from experiments using mice that there is a loss of immune function with age. Thus Callard, Basten and Blanden (1979) have related this loss to an abnormality in lymphocyte membranes while other workers suggest that this loss of immune competence is due to impaired regulation of autoantibody production (Szewczuk and Campbell, 1980).

Gupta and Good (1979) examined human PBL in young and ageing individuals using a number of surface markers. No significant difference was observed in absolute lymphocyte counts or in the percentages of T, B or third population lymphocytes. The T_γ

population was significantly increased and this increase was most noticeable in aged females. A concomitant decrease in T_{μ} was observed. Clot, Charmasson and Brochier (1978) showed that T cells increased from infants (3 months) to adults (20-50 years) and then decreased with ageing (75-97 years). Both Clot and colleagues (1978) and Brohee and associates (1980) demonstrated increased active E rosettes in aged individuals. Mellbye and co-workers (1972) found no significant difference in sIg positive cells between ageing humans and children while Clot, Charmasson and Brochier (1978) showed that sIg positive cells were low in adults and returned to the original levels with ageing; C3R positive cells decreased from the new born to aged individuals (Clot, Charmasson and Brochier, 1978).

Lymphocytes from aged individuals show normal responses to the non-specific mitogens PHA, Con A and PWM. However a lack of Con A-inducible suppressor cells was demonstrated (Abe et al, 1981). NK but not K cell activity was shown to be grater in males than females and greater in young individuals when compared to aged individuals (Penschow and MacKay, 1980).

Drug Treatment

It is not possible to present a comprehensive account of drug-induced effects on lymphocytes in this short section but a few examples relevant to the numbers and possible functions of Fc-receptor bearing cells are given below.

The majority of drug-induced changes in lymphocyte populations

have been noted with 'second-line' treatment (e.g. gold, penicillamine, immunosuppressive or steroidal drugs) and many of these effects have been shown in vitro. Thus D-penicillamine and copper salts can inhibit helper T cell function (Lipsky and Ziff, 1978; 1980), gold treatment can suppress lymphocyte transformation but not ADCC (Davis, Percy and Russell, 1979) and methyl prednisolone can affect suppressor cell function (Hirschberg et al, 1980). B cells have been shown to be highly sensitive to azathioprine and cyclophosphamide and these immunosuppressive drugs may also effect subpopulations of T cells (Dimitriu and Fauci, 1978; Hurme, 1979).

Some studies have been done whereby surface markers were employed. Thus dexamethasone treatment of a progranulocytic cell line, HL-60 reduced the number of Fc receptors per cell by 35-50% (Crabtree et al, 1979). Levamisole, administered in a single oral dose to healthy individuals, induced suppressor activity in the non-T_γ subset of T lymphocytes (Moriya et al, 1979). Low mitogen responses in patients with RA were restored by in vivo levamisole treatment; similarly treated adults showed no significant increase in responsiveness (Scheinberg et al, 1978).

Because of the effects of 'second-line' drugs and ageing on lymphocyte numbers and functions, the majority of patients studied and described in this thesis were receiving non-steroidal anti-inflammatory drugs (NSAIDs) unless otherwise stated and were age and sex-matched with controls. Nevertheless some non-steroidal therapy has been shown to affect lymphocyte functions. In vitro indomethacin can enhance the PHA response of rheumatoid peripheral blood but not synovial fluid lymphocytes (Panayi and Corrigall, 1979).

It is obvious that any comparisons made with respect to differences in function or numbers of rheumatoid lymphcoyte sub-populations are subject to error. However by restricting comparisons to patients taking NSAIDs alone, the problems are slightly alleviated. In this study normal healthy individuals and patients with osteoarthritis or ankylosing spondylitis were used as control groups.

Osteoarthritis (OA)

(i) General

Osteoarthrosis is a disease in which joint cartilage is progressively destroyed and replaced by new bone. This new bone appears beneath the worn cartilage and as outgrowths or osteophytes. Osteoarthrosis is a common condition; the frequency increases with age and is found predominantly in women over the age of 55. Osteoarthrosis can result from joint trauma, congenital or metabolic disorders (e.g. congenital dislocation of the hip or alkaptonuria) or as a secondary condition in diseases like rheumatoid arthritis and gout (Swinson and Swinburn, 1980).

The term osteoarthrosis is synonymous with degenerative joint disease (DJD) and suggests a purely mechanical 'wear and tear' condition devoid of inflammation. However there is morphological evidence to suggest that cartilage changes in DJD differ from those of the ageing process (Sokoloff, 1980) and many patients with DJD suffer episodes of joint inflammation. Originally these inflammatory osteoarthritides were described as disease variants but the high

incidence of inflammation in patients with DJD argues the use of the term osteoarthritis (OA) (Huskisson et al, 1979). Both pyrophosphate (McCarty, 1975) and hydroxyapatite crystals were detected in OA joint fluids and may be the cause of inflammation (Dieppe et al, 1976).

Since OA is not considered to be an auto-immune disease, patients with OA are often used as control subjects in studies using RA patients.

Aetiological Considerations

As previously stated, OA is considered to result primarily from joint injury. No synovial antigen was detected in a synovial membrane homogenate of three patients with OA but OA peripheral blood lymphocytes responded significantly to rheumatoid synovial antigen. The healthy control group showed no response. This reaction may reflect the inflammatory component of OA (Bacon et al, 1973). In contrast to RA, the anti-RANA antibody titre was within normal limits (Ferrell et al, 1981).

(ii) Cellular Aspects of Osteoarthritis

Synovial Membrane, Synovial Fluid and Peripheral Blood Cells

Scanning electron microscopy revealed that arthrosic synovial membranes (removed during total hip replacement) were infiltrated by numerous cells of small size. In contrast to the normal synovial membrane which presented a smooth, regular surface, arthrosic

membranes were covered with short flattened appendices (Gaucher et al, 1976).

Synovial fluid of osteoarthritic and rheumatoid joints were compared. Much lower numbers of leucocytes were detected in OA fluids and these were predominantly mononuclear cells; hydroxyapatite crystals were also found. In contrast, RA fluids contained no crystals and the predominant cell was the polymorphonuclear leucocyte.

Peripheral blood lymphocyte investigations have shown normal numbers of rheumatoid rosettes (Durance, Micheli and Fallet, 1974) and of Ia positive T cells (Yu et al, 1980).

Cellular Function

Cell mediated immunity is considered to be normal in that only one of six OA patients responded to native or heat aggregated IgG (Weisbart, Bluestone and Goldberg, 1975).

McGill and Twinn (1977) showed normal peripheral blood ADCC in a group consisting of 15 healthy and 15 OA subjects and Abdou and co-workers (1981) demonstrated normal suppressor activity in a group of 11 OA patients. Peripheral blood lymphocytes from OA patients suppressed healthy B cell Ig synthesis or secretion and the anti-suppressor cell antibody found in the serum of early RA patients was not detectable in OA sera.

Normal peripheral blood ADCC was inhibited by synovial fluids from 4 OA patients but the inhibition was less than that observed

using either rheumatoid fluid or fluids from patients with 'other connective tissue diseases'. The results were compared with inhibition by normal human serum and thus the increased inhibition by OA fluids may be an artefact of the method. Normal synovial fluid was not available (Cooke, Hay and Perumal, 1979).

Ankylosing Spondylitis

(i) General Introduction

Ankylosing spondylitis is a member of the seronegative spondarthritides, a group which also includes psoriatic arthritis, Reiter's disease and enteropathic arthritis.

The disease is characterised by sacroiliitis and spondylitis which eventually results in bone fusion (ankylosis). Ankylosing spondylitis occurs more commonly in males than females. In women, the age of onset is higher and cervical spine involvement less common. The hip joints are more usually affected in men. Features of ankylosing spondylitis are, in order of decreasing frequency of occurrence, peripheral joint involvement, iritis, urethritis, cardiac complications and pulmonary fibrosis (Gow, 1978; Swinson and Swinburn, 1980).

The aetiology of ankylosing spondylitis is unknown although Klebsiella has been cited as a possible infectious agent in susceptible individuals (Ebringer et al, 1977). The strong correlation of ankylosing spondylitis with the HLA-B27 antigen is well documented (Brewerton et al, 1973; Woodrow and Eastmond, 1978).

(ii) Lymphocyte Aberrations in Ankylosing Spondylitis

The majority of workers investigating lymphocytes in ankylosing spondylitis (AS) have studied peripheral blood lymphocytes. McDevitt and Bodmer (1974) questioned the relation of specific HLA antigens to disease and postulated that the association may be with abnormal immune responses. Nikbin and co-workers (1976) demonstrated a relationship between HLA-B27 and a diminished mixed lymphocyte response (MLR). Asymptomatic controls positive for HLA-B27 and AS patients showed similar MLRs which were significantly reduced in comparison with HLA-B27 negative controls. Weisbart and colleagues (1976) showed that peripheral blood leucocytes from patients with AS and their first degree relatives (24 or 36 individuals) reacted *in vitro* to autologous IgG. However some HLA-B27 positive individuals did not react and some individuals who showed the response were HLA-B27 negative. In this study, there was no relation between immune reactivity and the HLA-B27 marker.

Immunological reactivity in AS was demonstrated by Eghtedari, Davis and Bacon (1976); raised numbers of circulating immunoblasts (activated cells) were detected in some AS patients (11 of 39 cases). The plasma viscosity measurement, used as a parameter of inflammatory activity, was raised in all patients with increased levels of circulating immunoblasts.

T Lymphocytes

Reduced T cell numbers have been reported in AS peripheral blood in comparison with healthy individuals. This low T cell

population was independent of HLA-B27; both B-27 positive and negative AS patients had low numbers of T cells (Fan et al, 1977). Rosenthal and Muller (1975) and Nilsson and Biberfeld (1980), using neuraminidase treated sheep erythrocytes, found no difference in T cell numbers between AS and healthy individuals. Nilsson and Biberfeld (1980) suggested that the discrepancy with Fan's group may be a result of the enzyme treatment. However Froebel and associates (1979) found normal numbers of T cells when measured with neuraminidase treated and untreated sheep erythrocytes. In AS, raised levels of T_{γ} and normal numbers of T_{μ} were detected (Nilsson and Biberfeld, 1980).

Mitogen responses of PBL from patients with AS were normal when stimulated with optimal PHA doses (Fan et al, 1977; Nilsson and Biberfeld, 1980) or with a range of concentrations of PHA, Con A and PWM (Froebel et al, 1979).

Non-T Lymphocytes

Fan and associates (1977) detected normal numbers of C3R and FcR positive cells in the PBL of AS patients. In another study, both EA rosettes and sIg positive lymphocytes were reduced but this was not significantly different from normal (Froebel et al, 1979). Rosenthal and Muller (1975) also detected normal numbers of B and null cells in patients with AS. Wooley and Panayi (1978) measured the uptake of radiolabelled heat aggregated IgG by peripheral blood lymphocytes in a group of 14 patients of which 11 had AS. The remaining 3 patients exhibited symptoms of JCA. The group showed no differences in Fc binding from healthy controls.

Antibody-dependent cellular cytotoxicity to Chang cell targets was shown to be significantly reduced in the PBL of AS patients (Froebel et al, 1979). Panayi (1976) detected normal low levels of anti-Chang antibody in AS.

SECTION III

Fc Receptors (FcRs)

The presence of a receptor for the Fc region of immunoglobulin on lymphoid cells has been described in previous sections. However such receptors are detected on a wide range of cell types for all classes of immunoglobulins. In this section FcR positive human cell types will be described, FcR heterogeneity and specificity of human lymphocytes outlined and the possible functions of FcR positive cells presented. Table 1.3 summarises the data given in this section concerning the class and IgG subclass specificity of human peripheral blood leucocyte FcRs and their susceptibility to proteolysis. Table 1.4 shows the various functions ascribed to human leucocytes.

(i) FcR Positive Human Cells

Lymphocytes

Human FcR positive T and non-T lymphocytes have been described in Section I and therefore this section will briefly outline the class and subclass specificity for immunoglobulin and omit the functional aspects of $Fc_{\gamma}R$ and $Fc_{\mu}R$ positive lymphocytes previously described. Peripheral blood human lymphocytes selectively bind human monomeric IgG1 and IgG3 myeloma proteins. However, when aggregated, all subclasses of IgG are cytophilic for lymphocytes (Lawrence, Weigle and Spiegelberg, 1975).

Table 1.3

HUMAN LEUCOCYTE FcR SPECIFICITY AND SUSCEPTIBILITY TO PROTEOLYSIS

CELL	Ig CLASS	SUBCLASS of IgG	PROTEOLYSIS	
			TRYPSIN	PRONASE
T_γ	G	1,3	sensitive	sensitive
T_μ	M		sensitive	sensitive
B	G,D	1,3	sensitive	sensitive
NULL	G	1,3	resistant	sensitive
K	G	1,3	sensitive *	resistant *
NK	G	1,3	resistant *	
MACROPHAGE MONOCYTE	G	1,3	resistant	
NEUTROPHIL	G,A	1,3,4	resistant	

* cytotoxic ability measured rather than IgG binding

The NK cell does not require FcRs for activity

Table 1.4

FUNCTIONS OF FcR BEARING HUMAN LEUCOCYTES

CELL	FUNCTIONS					
	ACCESSORY	HELP	SUPPRESSION	CYTOTOXICITY		PHAGOCYTOSIS
				ADCC	SCMC	
T_γ			✓	✓	✓	
T_μ		✓	(✓)			
B			(✓)			
NULL	✓		✓	✓	✓	
K *				✓		
NK *					✓	
MACROPHAGE } MONOCYTE }	✓		✓	✓	✓	✓
NEUTROPHIL				✓	✓	✓

* K and NK cells are defined by their functional capacity but are included since both cell types bear Fc Rs

T Lymphocytes

As reported in Section I, human peripheral blood T lymphocytes express Fc_{μ}Rs or Fc_{γ}Rs or neither receptor. Receptors specific for IgD (Sjöberg, 1980) and IgA have also been detected (Lum, Beneviste and Blaese, 1980). The Fc_{α} receptor positive cells showed a lower MLC response than T_{γ} or T_{μ} and were less effective as helpers in the response to PWM. Yodoi and Ishizaka (1980) have demonstrated induction of $Fc_{\varepsilon}R$ *in vitro* by IgE in mixed lymphocyte culture. Hoover and colleagues (1981) showed specific FcR expression in myeloma patients; patients with IgG myeloma showed increased T_{γ} cells while IgA myeloma patients had high levels of T_{α}. Both sets of patients had normal levels of non-T $Fc_{\gamma}R$ and $Fc_{\alpha}R$.

The T_{γ} Fc receptor has been isolated; the molecular weight, measured using sodium dodecyl sulphate polyacrylamide gel electrophoresis under non-reducing conditions, was found to be 120,000 daltons (Cunningham-Rundles et al, 1980).

Non-T Lymphocytes

Receptors for IgG have been reported on B and null cells (see Section I) but are lacking on blast transformed B cells (Jondal, 1974). The $Fc_{\gamma}R$ of B cells has been extracted from the cells of a patient with chronic lymphocytic leukaemia. This had a molecular weight of 35,000 daltons and expressed phospholipase A2 activity (Suzuki et al, 1980; Suzuki, 1981). Receptors for IgD ($Fc_{\delta}R$) are also present on non-T cells (Sjöberg, 1980).

Null cells, isolated using anti-rhesus antibodies, were found to bind only IgG and not IgA, M, D or E. Of the IgG subclasses IgG1 and 3 bound with equal affinity while IgG2 and 4 showed very

weak binding (Frøland et al, 1974). Natvig and Frøland (1976) screened three anti-rhesus antibodies and showed that all three detected third population cells. The workers suggest that the suitability of anti-rhesus antibodies for the detection of this population may be related to the fact that they are primarily IgG1 and 3 subclass proteins, the two IgG subclasses that react most strongly with the $Fc_{\gamma}R$ of third population cells.

Neutrophils

The FcRs of human peripheral blood neutrophils have been shown to be specific for IgG and IgA and do not bind IgM, D or E (Henson, 1977). Lawrence, Weigle and Spiegelberg (1975) demonstrated binding by all subclasses of aggregated IgG and IgA. However, in unaggregated form, binding was achieved with IgG1, 3 and 4, IgA1 and 2 and secretory IgA. The authors suggest that $Fc_{\gamma}R$ and $Fc_{\alpha}R$ are distinct on neutrophil membranes.

Alexander, Titus and Segal (1979) measured the $Fc_{\gamma}R$ density of human peripheral blood leucocytes; neutrophils exhibited a high receptor density. Henson (1977) reported neutrophil FcRs to be trypsin and neuraminidase resistant.

Monocytes and Macrophages

Human monocytes and macrophages specifically bind IgG (Dorrington, 1977) and show subclass preference for unaggregated IgG1 and 3 (MacLennan, 1972; Lawrence, Weigle and Spiegelberg, 1975;

Dorrington, 1977). No $Fc_{\varepsilon}R$ have been reported (Hellstrøm and Spiegelberg, 1979) and IgA subclasses demonstrated insignificant binding (Lawrence, Weigle and Spiegelberg, 1975). In other mammalian species the binding is not IgG specific; there are reports of IgE binding by rat macrophages and monomeric IgM binding by guinea pig cells (Dorrington, 1977).

Platelets

The $Fc_{\gamma}R$ is present on human platelets and may be responsible for platelet injury induced by staphylococcal infection (Hawiger et al, 1979). It has also been suggested that the platelet $Fc_{\gamma}R$ is closely associated with the site for von Willebrand factor, which causes agglutination of platelets, since agglutination can be inhibited 'in vitro' by the addition of Fc or heat aggregated IgG but not by monomeric IgG, $F(ab')_2$, Fab or pFc (Moore, Ross and Nachman, 1978).

Basophils and Mast Cells

These cells have been shown to have receptors for IgE (Dickler, 1976 (b); Dorrington, 1977). Basophils bind IgG1 and 3 also (MacLennan, 1972).

Virus-Induced FcR Positive Cells

Cytomegalovirus and Herpes viruses can induce FcR expression on infected cells (Burns and Allison, 1977). The FcR induced by

Herpes Simplex Virus has been most studied and found to be trypsin sensitive while neuraminidase enhances IgG binding; receptor synthesis is inhibited by 2-deoxy-D-glucose and it is suggested that the receptor is possibly a glycoprotein. Anti-β_2 microglobulin did not inhibit IgG binding (McTaggart et al, 1978).

Robinson and co-workers (1977) evaluated lymphocyte surface receptors before and after transformation with Epstein Barr Virus (EBV). Initially, the distribution of cells with characteristics of B lymphocytes was similar in human umbilical cord blood and in the peripheral blood of cotton-top marmosets and woolly monkeys. However, after EBV infection, the transformed cells showed different surface markers; human cord lymphocytes expressed the complement receptor but not the FcR, woolly monkey cells were complement and Fc receptor positive and marmoset cells exhibit neither marker. Thus, transformation may increase or negate Fc receptor expression.

Cell Lines

Cell lines are usually derived by virus transformation or by 'in vitro' cell cloning or from patients with immunodeficiencies. Receptors for IgG and IgM have been reported (Preud'homme et al, 1979) and Hellstrφm and Spiegelberg (1979) described high $Fc_\epsilon R$ positive cells in a patient with chronic lymphocytic leukaemia.

Huber and colleagues (1976) tested a variety of cell lines for several membrane markers including FcR expression. Lymphoid cell lines demonstrated a B cell marker pattern, having sIg, C3

receptors (C3R) and low FcR density. The Burkitt's Lymphoma (BL) cell lines (i.e. EBV genome positive) demonstrated sIg, C3R and FcR while the non-BL cell lines showed varying degrees of expression of the three markers, two lines expressing sIg only. Myeloma cell lines were C3R and FcR negative while leukaemia cell lines were sIg negative but displayed C3R and FcR in variable concentrations. All FcR positive lines showed preferential binding of IgG 1 and 3.

Epithelial Cells

The placental plasma membrane bears $Fc_{\gamma}Rs$ and again binding of IgG 1 and 3 is more effective than 2 and 4 (van der Muelen et al, 1980).

Tonsil lymphocytes have been shown to bear receptors for IgM (Pichler and Knapp, 1978), IgG (Pichler and Broder, 1978) and IgE (Hellstrøm and Spiegelberg, 1979). Adenoid and spleen lymphocytes were also shown to bear $Fc_{\varepsilon}R$. The majority of $Fc_{\varepsilon}R$ positive lymphocytes are sIgM and sIgD positive (Hellstrom and Spiegelberg, 1979).

In contrast, Alexander, Titus and Segal (1979), using radiolabelled affinity cross-linked trimers of rabbit IgG, found no significant detectable $Fc_{\gamma}R$ on tonsil or thymus leucocytes. Most of the Fc receptors of splenocytes were on myeloid cells and monocytes. The discrepancies may be directly related to the different methods of detection.

(ii) Heterogeneity of Fc_{γ}Receptors on Human Lymphocytes

A number of different techniques have been employed to demonstrate FcR heterogeneity. Surface markers are either used alone or in conjunction with proteolysis or temperature changes.

Enzyme Treatment

Lobo and Horwitz (1976) examined the ability of B and L lymphocytes to bind aggregated IgG (agg G) or IgG complexed to erythrocytes (Ripley EA rosettes; EA_{Ri}). L lymphocytes bound both human and guinea pig agg G, formed Ripley rosettes and possessed trypsin resistant Fc receptors. B lymphocytes did not form Ripley rosettes and agg G binding was variable; trypsin treatment of B lymphocytes abolished the binding capacity.

Gormus, Woodson and Kaplan (1978a) measured the FcR expression of human mononuclear cells by Ripley rosettes, sensitised chicken erythrocyte rosettes (EA_{Ch}) or human agg G binding. Receptors for sheep erythrocytes (E rosettes) and C3 were also evaluated and sIg positive lymphocytes assessed. Measurements were made before and immediately after enzyme stripping and again following overnight culture. Pronase increased EA_{Ri} and EA_{Ch}, halved the number of E rosettes and almost abolished aggregate binding. No rosettes were noted before or after stripping when unsensitised or $F(ab')_2$ sensitised chicken erythrocytes were used. Following culture, similar results were seen irrespective of which enzyme was used for proteolysis; normal percentages of EA_{Ri} were detected but there was no significant regeneration of agg G receptors. These workers suggest

that heterogeneity exists among lymphocyte FcRs since they differ in susceptibility to proteolysis and re-expression and that lymphocytes may have unexpressed receptors which are exposed by pronase. Horwitz and Lobo (1975) also showed receptors for agg G to be pronase labile but reported regeneration of receptors following culture; these workers used lower pronase concentrations.

Three types of FcR positive cells were characterised by immunofluorescent studies using rabbit allotype/anti allotype complexes (Winfield, Lobo and Hamilton, 1977). Pronase sensitive, trypsin resistant cells formed 10-15% of peripheral blood lymphocytes and were identical to EA_{Ri} forming cells. Cells with low density FcR comprised 50% of the sIgM positive cells and $\sim$10% of T cells while the very low density FcR positive cells comprised $\sim$15% of peripheral blood T and B cells. All T and B cells were pronase and trypsin sensitive. Pichler and Knapp (1978) have shown tonsil B lymphocytes to be trypsin and pronase sensitive also.

Winchester and associates (1979) used six different immune complex test systems for the detection of FcRs. No one system was optimal for FcRs on all types of lymphocytes. Thus IgG/anti-IgG complexes and agg G gave highest binding of non-T cells while T_γ cells reacted better with ovalbumin/anti-ovalbumin and Keyhole Limpet haemocyanin (KLH)/anti-KLH complexes. Digestion with graded concentrations of pronase revealed that FcRs demonstrable by sensitised ox erythrocytes and KLH/anti-KLH complexes were more susceptible to digestion than those detected by IgG/anti-IgG complexes. The authors suggest that FcRs on T and null cells differ from those on B cells. Clements and Levy (1978) used four methods of FcR detection

and concluded that B lymphocytes require higher concentrations of IgG for detection.

It is apparent that some confusion exists in the literature although most workers agree that B and null lymphocyte $Fc_{\gamma}Rs$ differ both in their ease of detection and their susceptibility to proteolysis.

Another enzyme which is often used in the separation of T and B cells by E rosette depletion is neuraminidase. Increasing concentrations of this enzyme have been shown to selectively deplete IgMFcR and increase IgGFcR. The $Fc_{\mu}R$ is re-expressed after incubation; this re-expression is a result of active synthesis since it is prevented by cycloheximide. Neuraminidase treated T_{μ} lymphocytes can express $Fc_{\gamma}R$ (Schulof et al, 1980).

Other Methods Demonstrating FcR Heterogeneity

Human lymphocyte Fc heterogeneity with β_2 microglobulin (β_2m) has been demonstrated. Partial inhibition of agg G binding by anti-β_2m occurred on human tonsil and peripheral blood lymphocytes (Grey et al, 1975). Other workers have demonstrated EA rosette inhibition by anti-β_2m (Sarmay, Sanderson and Ivanyi, 1979). Enhanced EA rosette formation was achieved by the addition of β_2m and this enhanced binding was attributed to T_{γ} cells. Addition of anti-β_2m abrogated the response (Birch, Fanger and Bernier, 1979).

Temperature shift experiments have distinguished two types of $Fc_{\gamma}R$ on peripheral blood mononuclear cells. Type FcRI is shed

following a temperature shift from 4°C to 37°C and is located on FcR positive cells with a low cellular avidity to EA while FcRII is not shed following temperature shift. The shed FcRs interact with IgG but not IgM (Sándor et al, 1978; 1979).

(iii) Functional Capacity of FcR Positive Cells in Man

The most easily demonstrable and thus the most extensively studied function of FcR positive cells is that of antibody dependent cellular cytotoxicity (ADCC). Macrophages, monocytes and K lymphocytes are all capable of lysing antibody coated target cells and all bear FcRs. Monocytes active in ADCC have been shown to be of large size (Norris et al, 1979). Kurlander (1980) demonstrated that IgG can inhibit the in vitro monocyte mediated lysis of IgG coated erythrocytes but monocyte function is restored following washing. There is cross-reactivity between IgG from different species that induce K cell activity; for example, guinea pig IgG can induce ADCC by human K cells (Perlmann et al, 1975).

It has been suggested that ADCC is an in vitro phenomenon. MacLennan and co-workers (1973) showed that fresh human serum did not inhibit K cell activity against Chang Cell targets; however, monomeric IgG isolated by salt precipitation and DEAE chromatography blocked ADCC, and heat aggregation only slightly increased the blocking capacity. All four subclasses of human IgG inhibited K cell lysis of Chang liver cells; similar results were obtained using chicken erythrocytes although IgG 2 and 4 were found to be less effective inhibitors than IgG 1 and 3 (Spiegelberg, Perlmann and Perlmann, 1976). No inhibition was noted with human $F(ab')_2$

(Larsson, Perlmann and Natvig, 1973). MacLennan, Connell and Gotch (1974) showed that K cells specifically bound the CH3 region of IgG since anti-Chang Facb but not anti-HSA Facb inhibited K cell activity to Chang cell targets. Ziegler and Henney (1977), in contrast to MacLennan and associates (1974) found that, using Chang cell targets, aggregates were much more effective inhibitors of ADCC than monomeric IgG; incubation of lymphocytes with aggregated IgG followed by washing still produced marked inhibition. The order of effectiveness was as follows: cell associated immunoglobulin (Ig) $\geq$ immobilised immune complexes (i.c.) $>$ insoluble i.c. $>$ soluble i.c. $>$ heat aggregated IgG (agg G) $\gg$ monomeric Ig.

Gormus, Woodson and Kaplan (1978b) showed that incubating lymphocytes with pronase or papain produced no significant inhibition of ADCC against chicken erythrocytes whereas agg G almost totally abolished the ADCC of untreated lymphocytes and diminished the ADCC of enzyme treated cells. These authors concluded that peripheral blood lymphocytes active in ADCC have agg G and EA receptors and that K cells deficient in a functional agg G FcR (enzyme treated) can induce normal ADCC. As previously reported, Schulof and colleagues (1980) demonstrated selective depletion of $Fc_{\mu}R$ and enhanced expression of $Fc_{\gamma}R$ following neuraminidase treatment of human lymphocytes; however, this modulation of FcR phenotypes had no effect on ADCC against K562 target cells.

Spontaneous cell mediated cytotoxicity (SCMC) is effected by natural killer (NK) cells which also bear $Fc_{\gamma}Rs$ (Santoli et al, 1978; Gupta et al, 1978; Kay and Horwitz, 1980). However, the FcRs on NK cells are not required for cytotoxic activity since it

has been shown that treatment with trypsin or the addition of protein A will block ADCC but not SCMC (Kay, Bonnard, West and Herberman, 1977); interaction of lymphocytes with immune complexes (modulation) also abolishes ADCC but not SCMC (Pape, Moretta, Troye and Perlmann, 1979). Murine NK cells express a Ly5 phenotype and it has been postulated that interferon, produced by Ly5 positive cells, acts on Ly5 negative precursor cells to induce their differentiation into functional NK cells (Minato et al, 1980). Similar conclusions have been drawn following experimentation with human lymphocytes (Saksela, Timonen and Cantell, 1979). Supernatants of human lymphocyte/target cell co-cultures augmented the NK activity of virgin lymphocyte populations. Interferon was detectable in the supernatants and was found to induce natural cytotoxocity in a subpopulation of lymphocytes devoid of 'mature' NK cells.

FcRs have been implicated in the regulation of immune responses. Using murine spleen cells, Ryan and others (1975) showed that lipopolysaccharide (LPS) could not stimulate mitogenesis in the presence of immobilised IgG-IgG complexes. No similar inhibition was noted with immobilised IgG-$F(ab')_2$ complexes, soluble complexes or soluble IgG. A direct competition with FcR did not occur since pre-incubation with mitogen did not inhibit binding of agg G. These workers suggest that complex binding may produce an alteration of the cell membrane so that LPS cannot bind or may cause unresponsiveness. Oberbarnscheidt and Kølsch (1978) demonstrated that the anti-sheep red blood cell response of murine spleen cells could be suppressed by the addition of specific antigen/antibody complexes; a lack of suppression was shown when the $F(ab')_2$ fraction of anti-

body was used. The suppressive effect was shown to be T-cell independent indicating that the B cell FcR can influence B cell IgM antibody production.

In contrast to their suppressive effects, FcRs also appear to have accessory function. Carvalho, Davis and Horwitz (1980) noted a requirement for FcRs in the enhanced blastogenic response to KLH-anti KLH complexes by sensitised human lymphocytes and postulated the presence of an FcR bearing accessory cell. Lobo (1981) showed that positive or negative influences can be mediated by the same cell depending on the state of Fc-receptor stimulation; L cells modulated by immune complexes had suppressor effect while unmodulated cells enhanced immunoglobulin synthesis. Thus FcR bearing cells appear to be important regulators of Ig synthesis.

Phagocytosis by both neutrophils and monocytes appears to be mediated, at least in part, by FcRs. Monocyte phagocytosis is inhibited by heat aggregated IgG, IgG1 and IgG3 while $F(ab')_2$ fragments and IgG2 and 4 have no effect (Larsson, Perlmann and Natvig, 1973). The FcR involved in this inhibition is not species specific; guinea pig IgG1 and 2 can induce human monocyte phagocytosis in monomeric form and inhibit phagocytosis when complexed. This may be related to receptor heterogeneity (Øhlander, Larsson and Perlmann, 1978). Neutrophil phagocytosis can be blocked by monomeric IgG1 and 3 (Henson, 1977). In aggregated form IgG1, 2 and 3 will inhibit phagocytosis; aggregates of intermediate size eluted from a Sepharose 6B column were found to be the most efficient in blocking neutrophil phagocytosis (MacLennan et al, 1973).

The data provided in this and previous sections is summarised in table 1.4.

(iv) Characterisation of Fc Receptors and Immunoglobulin Binding

Owing to the heterogeneity of Fc receptors, previously described, it is not surprising that characterisation of the receptors has yielded inconsistent results. The receptor has been described as glycoprotein or lipoprotein in nature and molecular weights ranging from 15,000 to 130,000 daltons have been quoted. Controversy has also arisen concerning the site of attachment of the immunoglobulin molecule itself; different cells appear to bind different immunoglobulin domains. A few examples of FcR characterisation and FcR binding will be given in this section.

Wilkinson (1977) incubated several cell types with sphingomyelinase C and other lipid-specific agents; the binding of antibody coated chicken erythrocytes to human lymphocytes and monocytes and the binding of neuraminidase treated EA to human neutrophils was inhibited. It is apparent that membrane lipids are important constituents of the binding sites for Fc fragments on human leucocytes and sphingomyelin may play an important role in binding. Yagawa, Onoue and Aida (1979) isolated guinea pig peritoneal macrophage FcRs and found that phospholipase C (PLC) inhibited the activity of isolated receptors; these workers suggest that phospholipids may be required as structural supports for FcR activity. Suzuki (1981) has shown that the human B cell FcR has bound phospholipid and exhibits phospholipase A_2 activity.

Anderson and Grey (1977) investigated the FcRs of guinea pig and mouse macrophages, various mouse cell lines and mouse thymocytes and splenocytes. Detergent cell lysates were assayed for FcR activity and a strict correlation was found between the lysates and intact cells in terms of agg G binding. Enzymes which inhibited IgG uptake (PLC, papain, trypsin) also inhibited soluble FcR activity. In all cell lysates except those of mouse macrophages, isolated soluble FcR density was similar to that of serum high density lipoproteins. Phospholipase D and A did not alter soluble FcR activity. The authors postulate a lipoprotein nature for FcRs rather than steric hindrance by lipid associated membrane components.

The use of enzymes has led to the discovery of multiple FcRs on single cell types. Thus mouse macrophage cell lines have been shown to express a trypsin sensitive FcR and a trypsin resistant FcR which binds complexed rabbit IgG; monomeric rabbit IgG did not bind to the trypsin resistant receptor while the trypsin sensitive receptor bound both monomeric and complexed murine IgG 2a (Unkeless, 1977). More recently, a third FcR has been identified on mouse macrophages; this is specific for mouse IgG 3 and is also trypsin resistant (Diamond and Yelton, 1981).

The Fc region of immunoglobulin is composed of two domains, C_H2 and C_H3 (Fig. 3.1). It is generally accepted that the C_H2 domain is the site of complement fixation (Connell and Porter, 1971) but it has also been suggested that this domain may be involved in binding to FcRs.

Klein and colleagues (1977), using activated murine T cells

as the source of FcR, showed that the major site of binding on the IgG was localised to the $C_\gamma 3$ domain. However IgG and purified $C_\gamma 3$ and $C_\gamma 2$ fragments all bound but $F(ab')_2$ fragments did not. It was concluded that there may be a co-operative inter-domain site produced through quarternary binding. Other workers, using guinea pig lung macrophages, showed that IgG and Facb sensitised erythrocytes were equally effective in forming rosettes and papain derived $C_\gamma 3$ fragments did not inhibit the binding of IgG sensitised erythrocytes. Two possibilities were suggested, either both domains contribute contact residues or one domain stabilises the binding site of the other (Ovary, Saluk, Quijada and Lamm, 1976).

Human leucocyte FcR binding sites have been studied. Spiegelberg (1975) obtained an IgG1 myeloma protein with a deletion in the C_H3 domain. This protein formed two half molecules consisting of one heavy and one light chain. These half molecules did not bind C1q or bind to the FcRs of human lymphocytes, neutrophils or monocytes or guinea pig mast cells. However when aggregated, complement fixation and binding to lymphocytes, monocytes and neutrophils was observed. It was postulated that IgG1 may have several (at least two) sub-molecular binding sites which are localised on the $C_\gamma 2$ and $C_\gamma 3$ domains. Proteins with both sites could bind to C1q and FcRs in unaggregated form whereas proteins having a site on only one domain would need to be aggregated in order to bind. Ciccimara, Rosen and Merler (1975) also utilised myeloma proteins with deletions. They found that the primary site of attachment of human monocytes was in the C_H3 domain since myeloma IgG1 and 3 proteins with deletions in the C_H2 region showed unimpaired ability to bind. The binding site was localised to a ten amino acid peptide within the $C_\gamma 3$ domain.

In contrast, Alexander, Andrews, Leslie and Wood (1978) showed that pepsin derived pFc' fragments poorly inhibited either EA rosette formation or uptake of ^{125}I labelled human IgG by human monocytes.

Ripley rosettes were strongly inhibited by Fc and even more strongly by the Fch fragments of IgG3. No inhibition was observed when Fab, $F(ab')_2$ or pepsin derived pFc' fragments were added to the lymphocytes. Thus it appears that third population lymphocyte FcRs bind primarily the $C_\gamma 2$ region (Frøland et al, 1974). This is interesting since, as previously described, third population lymphocytes have K cell activity. MacLennan, Connell and Gotch (1974) found no inhibition of K cell activity when anti-HSA Facb was added suggesting that K cells bind primarily the $C_\gamma 3$ region of IgG. The myeloma protein, previously described, weakly inhibited K cell activity (Spiegelberg, Perlmann and Perlmann, 1976).

It is obvious from this brief section that the role of Fc receptors in immunological mechanisms is unclear. However the presence of these receptors on many cell types, their heterogeneity and ease of modulation, argues their importance. In rheumatoid arthritis there is an abnormal response to IgG and this reactivity has been attributed to the C_H2 region (Johnson and Faulk 1976; Hall, 1978). However it is possible that it is not the IgG which is altered but the binding site. In this thesis, abnormal binding of IgG by rheumatoid lymphocytes will be reported. Part of this work has been published elsewhere (Hall, Winrow and Bacon, 1980).

CHAPTER 2

MATERIALS AND METHODS

SECTION 1

MATERIALS

All chemicals were obtained from the British Drug Houses Ltd., Poole, unless otherwise stated.

All media reagents were obtained from Gibco Bio-Cult Ltd., Scotland, except those listed below.

MATERIALS	SUPPLIER
Bio Beads SM2 (20-50 mesh)	Bio-Rad Laboratories Ltd., Bromley, Kent
Carbonyl Iron Powder (type SF)	GAF (G.B.) Ltd., Wythenshawe, Manchester
Pokeweed Mitogen (lyophilised; crude preparation from Phytolacca americana)	Gibco Bio-Cult Ltd., Paisley, Renfrewshire, Scotland
Plasmin (human from Cohn fraction I; lyophilised)	Kabi Vitrum Ltd., Ealing, London
Concanavalin A (lyophilised; from the jack bean Canavalia ensiformis)	Pharmacia (G.B.) Ltd., Hounslow, Middlesex
Ficoll-Paque (density 1.077 $\pm$ 0.001 g/cm^3)	
Helix Pomatia Lectin-Sepharose 6MB	
Protein A - Sepharose 4B	
DEAE Sephadex A50	
Sephadex G100 (fractionation range 4,000 - 150,000 daltons)	
Sepharose CL 6B (fractionation range 10^4 - 4 x 10^6 daltons)	

MATERIALS	SUPPLIER
(Methyl - ^{3}H) Thymidine Specific activity 5 Ci/mmol 21 mCi/mg Radioactive concentration 1 mCi/ml	Radiochemical Centre Ltd., Amersham, Bucks.
Sodium Chromate (^{51}Cr) Solution Specific activity 250-500 μCi/μg	
Hyaluronidase (Type II; ovine testes; lyophilised)	Sigma Chemical Co. Ltd., Poole, Dorset
Neuraminidase (Type VI; Clostridium perfringens; lyophilised)	
Phospholipase C (Type I; Clostridium welchii; lyophilised)	
Pepsin (hog stomach mucosa; lyophilised)	
Papain (Papaya latex; x 2 crystallised; suspension in 0.05M sodium acetate pH 4.5)	
Trypsin (Type IX; porcine pancreas; lyophilised)	
N-acetyl-α-D-galactosamine	
N-acetyl-D-glucosamine	
α-L-fucose	
2-deoxy-D-glucose	
Calf Blood in Alsevers Solution (Animal No. 21)	Tissue Culture Services Ltd., Slough, Berks.
Sheep Blood in Alsevers Solution (both obtained fresh fortnightly)	
Hanks Balanced Salt Solution (x 10 concentrate)	Wellcome Reagents Ltd., Hither Green, London
Phytohaemagglutinin (purified from Phaseolus spp.; lyophilised)	

ROUTINE BUFFERS AND MEDIA

10/150

0.1M disodium hydrogen phosphate

0.1M potassium dihydrogen phosphate

The above solutions were mixed to pH 7.4 and the resulting phosphate buffer diluted 1 in 10, incorporating 8.77 g/litre of sodium chloride (150mM). The solution was stored at 4°C when not in use.

Calcium and Magnesium Free Salt Solution (CMFSS)

x 10 concentrate

	g/litre	
Sodium chloride	80.000	
Glucose	16.000	
Potassium chloride	4.000	
Potassium dihydrogen phosphate	0.600	
Disodium hydrogen phosphate	0.475	pH 7.3

The concentrate was filter-sterilised using a 0.45μm filter (Millipore) and stored at 4°C, being diluted immediately before use. The pH was corrected with IM sodium hydroxide solution if necessary.

Phosphate Buffered Saline (PBS)

x 10 concentrate

	g/litre
Sodium chloride	80.00
Potassium chloride	2.00
Disodium hydrogen phosphate	11.50
Potassium dihydrogen phosphate	2.00

The above concentrate was filter sterilised, stored at 4°C and diluted immediately before use incorporating :-

Calcium Chloride	0.1 g/litre	
Magnesium Chloride	0.1 g/litre	pH 7.4

The pH was adjusted if necessary

Lymphocyte Medium

	ml/litre	
RPMI 1640 (x 10 concentrate)	100.0	
200 mM glutamine (GLN)	20.0	
Penicillin/Streptomycin Solution (5,000 I.U./ml of each; P/S)	20.0	
Heat Inactivated New Born Calf Serum (HINBCS)	100.0	
7.5% Sodium Bicarbonate	27.0	pH 7.3

The medium was prepared using sterile distilled water, adjusted to pH 7.3 with sterile IM sodium hydroxide and stored at 4°C.

K562 Medium

	ml/litre	
RPMI 1640 (x 10 concentrate)	100.0	
200 mM GLN	20.0	
P/S	20.0	
Heat Inactivated Foetal Calf Serum (HIFCS)	150.0	
7.5% Sodium Bicarbonate	27.0	pH 7.3

Sterile distilled water was used in the preparation, the pH adjusted with sterile IM sodium hydroxide when necessary and the medium stored at 4°C.

Hanks Balanced Salt Solution (HBSS)

HBSS (x 10 concentrate) was diluted with sterile distilled water and brought to pH 7.4 with sodium bicarbonate solution or 4% tris hydroxymethyl methylamine (TRIS) solution.

Scintillation Fluid

To 2.5 litres of toluene was added :-

12.50 g	2,5 - Diphenyloxazole (PPO)
0.75 g	1,4 - Di-2-(4-methyl-5-phenyloxazolyl) benzene (POPOP)

SECTION II

METHODS

(i) PREPARATION, HEAT AGGREGATION AND ENZYMIC DIGESTION OF IMMUNOGLOBULIN G (IgG)

PREPARATION OF IgG

(a) AFFINITY CHROMATOGRAPHY - Protein A-Sepharose CL-4B (see Fig. 2.1)

Protein A is a protein isolated from the cell wall of Staphylococcus aureus and has been demonstrated to be a useful immunosorbent for the isolation of IgG (Hjelm, Hjelm and Sjöquist, 1972). Human IgG subclasses have been studied; only IgG3 is not bound by protein A (Kronvall and Williams, 1969). The binding is specific for the Fc region of the IgG molecule (Forsgren and Sjöquist, 1966; Kronvall and Fromell, 1970). More recently, Stewart, Varro and Stanworth (1978), using rabbit IgG enzymic cleavage products, were unable to demonstrate reactivity of either Facb or pFc' fragments with staphylococcal protein A; this property can be exploited in the preparation of Facb (described later in this section). Protein A can also interact with subclasses of IgG of species other than humans and rabbits (Goudswaard et al, 1978; Chalon, Milne and Vaerman, 1979) and may bind other human immunoglobulins (Brunda, Minden and Grey, 1979).

A commercial preparation of protein A, covalently coupled to Sepharose CL-4B by the cyanogen bromide method, was used for the majority of IgG preparations. The swollen gel contains 2 mg/ml of protein A with a binding capacity for human IgG of approximately 25 mg IgG/ml gel. A column (0.9 x 15 cm, Pharmacia K9), containing

Fig. 2.1

PREPARATION OF IMMUNOGLOBULIN G (IgG) AND ITS PLASMIN DIGESTION PRODUCTS BY COLUMN CHROMATOGRAPHY

Protein eluted from the columns was measured by absorbance at 280 nm. Typical elution profiles are shown opposite.

Legend

 material from first column applied to second column.

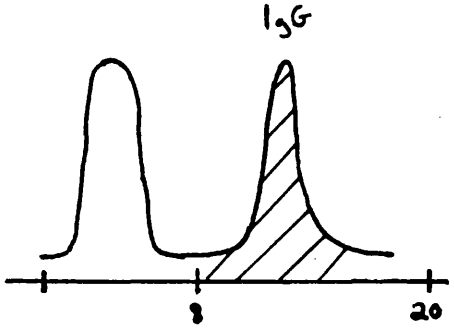
IMMUNOGLOBULIN G (IgG)

Immunoglobulin G was prepared by affinity chromatography on protein A-Sepharose 4B (PrA-4B) as described in chapter 2, section II (i). The IgG containing fractions (shaded) were pooled, concentrated and applied to a Sepharose CL-6B column (6B). Monomeric IgG was pooled and concentrated.

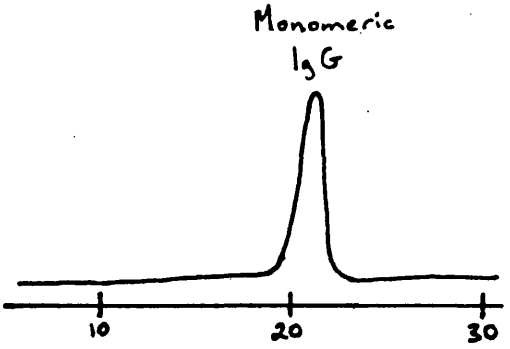
PLASMIN

IgG prepared by affinity chromatography (above) was incubated with human plasmin (chapter 2, section II (i) and the digest fractionated on Sephadex G100 (G100). The protein eluting in the void volume comprising Facb fragments and unreacted IgG (shaded) was concentrated and applied to an affinity column to purify the Facb fragments.

IMMUNOGLOBULIN G

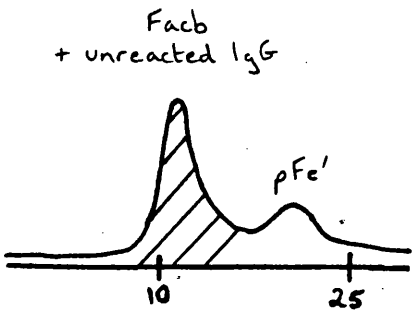


PrA-4B

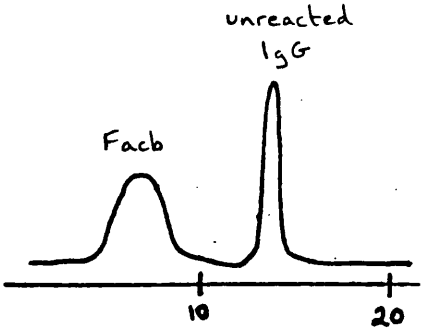


6B

PLASMIN



G100



PrA-4B

approximately 5 ml of swollen gel was prepared; serum (2-5 ml) was applied to the column and eluted with 10/150 to remove contaminating serum proteins. The bound IgG was then eluted with IM acetic acid and the acid fraction concentrated over an Amicon Diaflo XM50 membrane (nominal exclusion limit 50,000 daltons). All separations were carried out at a temperature of 4°C. The acid concentrate was then loaded on to a Sepharose CL6B column and eluted with 10/150 at a flow rate of 8 ml buffer/hour, collecting 4 ml fractions. Monomeric IgG fractions (assessed from an OD_{280} trace) were pooled, concentrated as above and total protein measured using the Folin-Ciocalteu method (Lowry et al, 1951).

(b) ION-EXCHANGE CHROMATOGRAPHY - Diethyl aminoethyl (DEAE) Sephadex

Myeloma sera, which may contain large amounts of IgG and so swamp the protein A column were separated using DEAE Sephadex by a modification of the method of Ishizaka and co-workers (1965).

DEAE Sephadex was swollen in 10 mM phosphate buffer pH 6.5, containing 0.01% sodium azide and stored at 4°C until required. A small amount of the suspension was filtered on a Büchner funnel and washed extensively with 10 mM phosphate buffer pH 6.5 without azide. The cake of Sephadex was added to the serum to form a paste and stirred for 1 hour at 4°C. The suspension was then filtered, using a Büchner funnel, and washed with the phosphate buffer. The filtrate was concentrated over an Amicon Diaflo membrane, as before, and dialysed against 10/150. Protein was assayed by the Folin Ciocalteu method.

In all cases, a small volume of the concentrate was applied to the Sepharose CL-6B column to check for aggregation. The yield was not as good as with the protein A column but, since myeloma sera were used, this was not a problem.

HEAT AGGREGATION OF IgG

Heat aggregated IgG (HAGG) was prepared by maintaining an IgG solution at 63°C for periods of time varying from 10 to 60 minutes. Aggregation times are quoted for individual experiments.

ENZYMIC DIGESTION OF IgG

(a) PREPARATION OF $F(ab')_2$ - PEPSIN (see Fig. 2.2)

Pepsin digestion was carried out according to Nisonoff Markus and Wissler (1961).

IgG was prepared on protein A Sepharose 4B (described previously in this section), dialysed against 0.01M acetate buffer pH 4.5 for 4 hours at 4°C and adjusted to 10-30 mg/ml. A solution of 1 mg pepsin/ml acetate buffer was prepared, added to the dialysate to give an enzyme/substrate ratio of 1:100 and the solution was incubated for 22 hours at 37°C. Pepsin was inactivated by the addition of solid Tris (hydroxymethyl) methylamine (TRIS) to a pH of 8.0 and the digest was fractionated on a column of G100 Sephadex, using 10/150 as eluant. The protein eluting in the void volume was pooled, concentrated and applied to the protein A Sepharose 4B; the unbound protein fractions ($F(ab')_2$) were pooled, concentrated and assayed for protein.

Fig. 2.2

ENZYMIC DIGESTION PRODUCTS OF IMMUNOGLOBULIN G (IgG) PREPARED BY COLUMN CHROMATOGRAPHY

Protein eluted from the columns was measured by absorbance at 280 nm. Typical elution profiles are shown opposite.

Legend

material from first column applied to second column

material from second column applied to third column

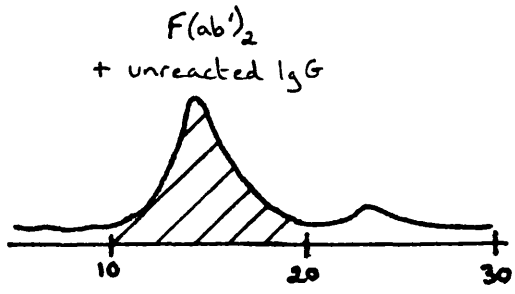
PEPSIN

A pepsin digest of IgG (chapter 2, section II (i)) was applied to a Sephadex G100 (G100) column. Unreacted IgG and $F(ab')_2$ fragments (shaded) were separated by affinity chromatography.

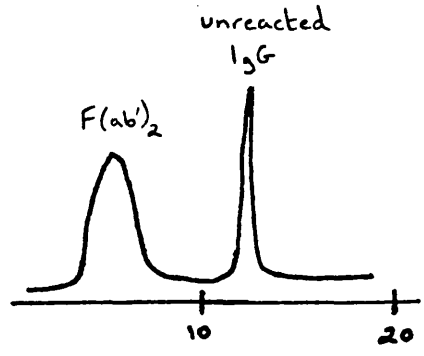
PAPAIN

IgG was treated with papain (chapter 2, section II (i)) and fractionated on Sephadex G100 (G100). The Fab and Fc fragments, being of similar molecular weight, fractionate together (shaded) and were therefore pooled and separated by affinity chromatography on protein A-Sepharose 4B (PrA-4B). Any unreacted IgG, which may peak with the fragments, will adhere to protein A together with the Fc fragments and may be removed by a second fractionation using Sephadex G100 (G100)

PEPSIN

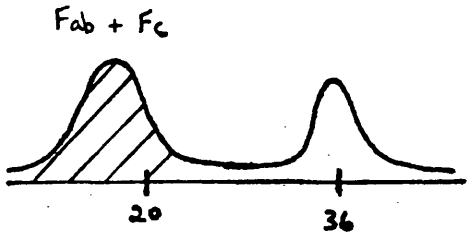


G100



PrA - 4B

PAPAIN



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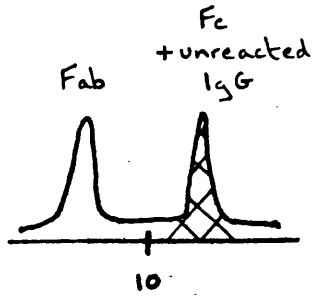

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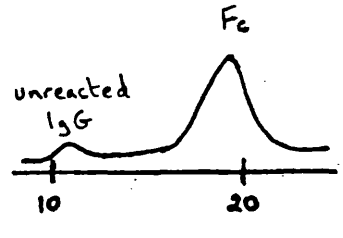


G100

(b) PREPARATION OF Fab and Fc - PAPAIN (see Fig. 2.2)

Papain digestion was carried out using a slight modification of the method described by Porter (1959).

IgG was prepared on protein A-Sepharose 4B. Ethylene diamine tetra acetic acid (EDTA) and cysteine were added to the IgG solution, the pH corrected to 7.0 with IM sodium hydroxide solution and the volume adjusted to give final concentrations of cysteine and EDTA of 0.01M and 0.002M respectively in 10-30 mg/ml of IgG solution. Papain was added to produce an enzyme/substrate ratio of 1:100 and the solution was incubated at 37°C for 16 hours. The enzyme reaction was terminated by the addition of iodoacetamide to 0.01M and the digest was applied to a G100 Sephadex column. Protein was eluted with 10/150 at 8 ml/hr and 4ml fractions were collected. The Fab and Fc fragments, being of similar molecular weight, are excluded from the column in the same fractions and must, therefore, be separated using the protein A-Sepharose 4B column, thus Fab fragments were eluted in the phosphate (unbound) fraction and Fc fragments in the acid (bound) fraction. The acid concentrate was further purified on G100 Sephadex to remove any unreacted IgG, if present.

(c) PREPARATION OF Facb AND pFc' - PLASMIN (see Fig. 2.1)

Facb and pFc' were prepared as described by Stewart, Smith and Stanworth (1973).

IgG was prepared by affinity chromatography on protein A-Sepharose 4B. The concentrated acid fraction was immediately adjusted to pH 2.7 with IM hydrochloric acid (HCl) and maintained at 37°C for 1 hour.

After incubation, the pH was adjusted to 7.0 with IM sodium hydroxide and human plasmin was added (3 CU/100 mg IgG). The solution was incubated at 37°C for 4 hours, with shaking, and the digest then applied to a Sephadex G100 column and eluted with 6M urea in 0.1M acetate buffer pH 4.6 at a flow rate of 8 ml/hr; fractions of 4 ml volumes were collected. The protein eluted in the void volume (i.e. Facb and unreacted IgG) was concentrated and dialysed against 10/150 for 4 hours at 4°C before loading on to the protein A-Sepharose 4B column. Since Facb does not bind to protein A (Stewart, Varro and Stanworth, 1978), this fragment was eluted in the phosphate fraction whilst residual IgG bound to the affinity column. The pFc' fragments were recovered from the G100 column and the concentrate dialysed against 10/150 before storage and use. Protein was assayed using Folin-Ciocalteu's solution.

The purity of the Facb preparation was checked using a standard Ouchterlony immunodiffusion method. A commercial donkey anti-rabbit whole serum was used and also an antiserum to rabbit pFc', kindly donated by Dr. I. M. Hunneyball (Boots Co. Ltd., Nottingham). The Facb preparation gave a single precipitin line against the anti-rabbit serum, but did not react with the anti-pFc'.

(ii) LYMPHOCYTE PREPARATION

(a) WHOLE BLOOD

Lymphocytes were separated by the isopaque ficoll method of Böyum (1968; 1976).

Heparinised venous blood (30 I.U. heparin/ml blood) was mixed 1:1 with CMFSS and carefully layered on to Ficoll-Paque in unsiliconised conical glass centrifuge tubes. Approximately 3 ml Ficoll-Paque was used to 6 ml of diluted blood. Diluting the blood helps to prevent aggregation of erythrocytes, thus eliminating trapping of lymphocytes and producing higher yields. The tubes were then centrifuged at 400 g in a temperature controlled MSE Mistral 2L centrifuge at 20°C for 30 minutes. During this period, erythrocytes, agglutinated by the ficoll, and granulocytes, which became dense due to ingestion of sodium diatrizoate, sedimented to the bottom of the tube while the lymphocytes, monocytes and platelets remained at the interface.

The lymphocyte rich interface layer was removed using a pasteur pipette, transferred to a clean centrifuge tube and washed three times with CMFSS by careful resuspension and pelleting at 400 g for 10 minutes.

(b) SYNOVIAL FLUID

Heparinised synovial fluid (30 I.U./ml fluid) was treated with hyaluronidase (0.15 NF Units/ml fluid) for 30 minutes at 37°C prior to dilution and separation as described for whole blood.

CARBONYL IRON TREATMENT

In some instances, whole blood was pretreated with carbonyl iron to produce a lymphocyte preparation depleted of mononuclear phagocytes. Carbonyl iron powder was added to whole blood at a concentration of

20 mg/ml and mixed for 30 minutes at 37°C before diluting 1:1 and separating on Ficoll-Paque as previously described. Care was taken to transfer as little carbonyl iron as possible to the Ficoll-Paque gradient to avoid trapping of lymphocytes. Using the above carbonyl iron concentration $< 0.5\%$ monocytes were present in the interface lymphocyte preparation judged by esterase staining (Section II (v)) of fixed smears (200 cells counted).

CELL COUNTS AND VIABILITIES

Counting fluid comprising 3% acetic acid/0.1% methylene blue was used for leucocyte counts. An aliquot of the cell suspension was diluted with counting fluid, causing lysis of erythrocytes, and cell numbers were enumerated using a Neubauer haemocytometer.

Viabilities were assessed by the trypan blue exclusion method. Viable cells exclude the stain but non viable cells are unable to do so and thus appear dark blue. The trypan blue technique is not a sensitive test of damage to lymphocytes since some non staining cells may appear, under phase contrast, to be in an advanced state of degeneration (Tennant, 1964).

(iii) ROSETTE TECHNIQUES

SHEEP ERYTHROCYTE (E) ROSETTES

(a) Materials

Sheep blood in Alsevers solution

Absorbed heat inactivated new born calf serum (AHINBCS) :-

To 1 ml of washed (x3) packed sheep blood

was added 9 ml HINBCS. The suspension was well mixed and maintained at 4°C for 16 hours. The serum was then spun at 400 g for 10 minutes and the supernatant stored in 0.5 ml aliquots at -20°C.

(b) Preparation of Sheep Blood

A small volume (~1ml) of sheep blood was washed three times (x3) with phosphate buffered saline (PBS), the final supernatant being removed carefully using a pasteur pipette. To 0.5 ml of AHINBCS was added 0.025 ml of packed cells and the volume was made up to 5 ml with PBS.

(c) Preparation of Neuraminidase Treated Sheep Erythrocytes (SRBC)

In some instances (e.g. rosette depletions), the sheep blood was treated with neuraminidase; this forms a more stable rosette. Sheep blood was washed three times (x3) in PBS as above and 0.05 Units of neuraminidase were added to approximately 0.5 ml of packed cells. The volume was made up to 5 ml with PBS and the solution incubated for 30 minutes at 37°C. Following a further three washes with PBS, the erythrocytes were prepared as above to produce a 0.5% v/v suspension in PBS containing 10% AHINBCS.

(d) Preparation of Rosettes

Washed lymphocytes, prepared by isopycnic centrifugation on Ficoll-Paque, were adjusted to 2 x 10^6/ml and equal volumes (200μl) of lymphocytes and SRBC suspension were mixed in stoppered, plastic

round-bottomed tubes (LP3-Luckham). Incubation at 37°C for 10 minutes was followed by centrifugation at 200 g for 3 minutes. The pelleted tubes were then maintained at 4°C for 2 hours before addition of 2 drops of a 0.2% solution of crystal violet in PBS. The tubes were resuspended for 1 minute using a rotary turntable (20 rpm; Baird and Tatlock Ltd.) and placed in a counting chamber (Neubauer). Lymphocytes (stained violet) surrounded by 3 or more erythrocytes were scored as rosettes and the percentage of rosetting cells was calculated from the observation of 200 lymphocytes.

When using neuraminidase treated erythrocytes, only 45 minutes incubation at 4°C was required for rosette formation.

COMPLEMENT (C3) ROSETTES

(a) Preparation of C3b coated erythrocytes

A 10% sucrose solution was prepared containing 0.008 g calcium chloride and 0.0085 g magnesium chloride in 500 ml. To 30 ml of this solution was added 3 ml of fresh blood from a healthy O rhesus negative donor and the suspension was mixed gently for 10 minutes at 37°C (rocking turntable). Red cells were washed x 3 with copious amounts of PBS and a 1% v/v suspension of erythrocytes in PBS was prepared (0.1 ml packed cells in 9.9 ml PBS).

The low ionic strength buffer enhances coating of erythrocytes with C3b (Stratton and Rawlinson, 1974; Freedman and Mollison, 1976). The cells were kept on ice when not in use.

(b) Rosettes

Lymphocytes at a concentration of 2 x 10^6/ml CMFSS and the 1% red cell suspension were mixed 1:1 in LP3 tubes, sedimented at 200 g for 3 minutes, incubated at 4^oC for one hour and the rosette preparations stained and counted as for E rosettes.

ERYTHROCYTE-ANTIBODY (EA) ROSETTES

(a) Materials

Calf blood in Alsevers solution

Rabbit anti-calf erythrocyte IgG

Prepared by affinity chromatography (Section II (i)) of rabbit anti-calf erythrocyte serum (see below).

(b) Preparation of anti-calf erythrocyte (RBC) serum

The antiserum was raised in rabbits by a number of different techniques :-

Repeated intravenous injections of washed calf RBC

Repeated intradermal injections of washed calf RBC emulsified 1:1 with Freund's incomplete adjuvant

Repeated intradermal injections of washed calf RBC with heat killed Mycobacterium tuberculosis

In all cases, the animals were bled after four weeks and the serum separated and frozen.

(c) Preparation of sensitised erythrocytes

Calf blood (0.5 - 1.0 ml) was washed three times with PBS, pelleted and the final supernatant removed carefully with a pasteur pipette. A 2% v/v suspension of calf erythrocytes was prepared (i.e. 0.2 ml packed cells in 10 ml PBS).

Antibody coated (sensitised) calf RBC were prepared by mixing equal volumes of the 2% calf RBC suspension and the required dilution of antibody solution and incubating at 37°C for 30 minutes. An optimum sub-agglutinating concentration was determined for each rabbit anti calf RBC IgG preparation and this was used for assessing total EA rosette numbers. The sensitised cells were sedimented at 200 g, washed three times with PBS and made up to volume with PBS resulting in a 1% suspension of antibody coated calf erythrocytes.

(d) Rosettes

Prepared, washed lymphocytes were adjusted to 2 x 10^6/ml CMFSS and equal volumes (100 - 200 µl) of lymphocytes and 1% sensitised calf erythrocytes were mixed in LP3 tubes sedimented at 200 g for 3 minutes and maintained at 4°C for one hour.

After incubation, crystal violet solution was added to the tubes and the pellets were resuspended and counted as for E rosettes.

Facb ROSETTES

Facb rosettes were prepared in the same manner as EA rosettes

using rabbit Facb anti calf RBC as the sensitising antibody (Hall, Winrow and Bacon, 1980; Section II (i)).

Sensitised erythrocytes were mixed with lymphocytes in equal proportions (100 - 200μl) in the same manner as EA rosettes and treated accordingly.

ROSETTE DEPLETION EXPERIMENTS

Mononuclear cells (10 - 20 x 10^6) were rosetted in the normal manner. After incubation at 4^{o}C for 60 minutes (EA and Facb rosettes) or 45 minutes (E rosettes), the pelleted cells were carefully re-suspended using a pasteur pipette, layered over ficoll/hypaque gradients and spun at 400 g for 30 minutes at 20^{o}C. Rosetted cells, by nature of their mass, formed a pellet at the base of the tube while non-rosetted cells accumulated at the interface. Rosettes were set up to analyse depleted and untreated lymphocyte populations.

After E rosette depletion, E rosette positive mononuclear cells can be reclaimed from the base of the tube since E rosettes can be disrupted by incubation at 37^{o}C. Pelleted cells were incubated at 37^{o}C for 20 minutes and then layered to to ficoll/hypaque and spun at 400 g for 30 minutes. The centrifuge buckets and tubes were warmed to 37^{o}C before use. Interface cells were harvested and rosettes prepared.

EA and Facb rosetting cells can be reclaimed by lysis of erythrocytes with 0.83% ammonium chloride but this may leave pieces

of erythrocyte membrane on the lymphocyte receptor.

(iv) RECEPTOR STUDIES

ENZYME TREATMENT OF LYMPHOCYTES

(a) Neuraminidase

Isolated, washed mononuclear cells were suspended in 2 ml of TRIS buffered Hanks balanced salt solution (TBH) containing neuraminidase at a concentration of 5 μg/ml and incubated at 37°C for 45 minutes as described by Hammerström and associates (1973). The cell suspension was then washed twice in PBS containing 0.2% human serum albumin and 0.02% sodium azide.

E, EA and Facb rosettes were set up before and after treatment with neuraminidase. Cell viabilities were assessed using trypan blue (Section II (ii)).

(b) Trypsin

Isolated, washed mononuclear cells at 4 x 10^6 cells/ml were incubated at 37°C for 30 minutes, washed with warm medium (HBSS containing 20% HINBCS) and subsequently incubated with 0.25% trypsin for 30 minutes at 37°C. Cells were then washed in warm medium.

Before setting up E, EA and Facb rosettes, trypsin treated and untreated cell populations were washed once in CMFSS and adjusted to a concentration of 2 x 10^6 cells/ml. Viable cells were enumerated using trypan blue.

(c) Phospholipase C

Phospholipase C at concentrations of 0.1 U/ml, 0.5 U/ml and 1.0 U/ml was incubated with isolated, washed mononuclear cells (4×10^6/ml) for 30 minutes at 37°C as described by Wilkinson (1977). Cells were then washed three times in CMFSS, viabilities assessed and EA and Facb rosettes were prepared.

Fc RECEPTOR SHEDDING

Mononuclear cell preparations were maintained at 4°C for 30 minutes and then immediately incubated at 37°C for 30 or 60 minutes (Sándor, Füst, Medgyesi and Gergely, 1978). The degree of receptor shedding was determined by setting up EA and Facb rosettes on shed and untreated populations. Supernatants of shed lymphocytes were stored at -20°C.

(v) CELL CHARACTERISATION STUDIES

BIO BEAD COLUMN CHROMATOGRAPHY

Styrene divynyl benzene beads (Bio Beads SM2) were prepared for use as described by Holloway (1973). Briefly, approximately 200 ml of methanol was added to 20 g of beads, the mixture stirred for 15 minutes using a glass rod and the copolymer beads collected on a sintered glass funnel (G3; pore size 15 - 40 μm), washed with a further 500 ml of methanol and, without drying, washed with one litre of distilled water. The moist beads were then slowly washed with 500 ml of 10/150 and stored at 4°C in 10/150 containing 0.2%

sodium azide until required. Immediately before use, the beads were washed in azide free 10/150.

The separation procedure used was a modification of the method of Pang and Wilson (1978). The washed beads were incubated at room temperature with 20% HINBCS in HBSS until the beads became orange in colour (approximately 1 hour). A Pharmacia K9 column (15 x 0.9 cm), with an 80 μm mesh base net, was packed with 8 ml of beads, eluted with several volumes of 20% HINBCS/HBSS and allowed to equilibrate at 37^{o}C for 30 minutes. Ficoll-Paque prepared lymphocytes (20 x 10^{6}) in 2 ml HBSS were applied to the column and incubated for 30 minutes at 37^{o}C before eluting with 30 ml HBSS.

E, EA and Facb rosettes were set up using cells before and after column separation.

HELIX POMATIA LECTIN - SEPHAROSE 6MB COLUMN CHROMATOGRAPHY

Helix pomatia lectin, prepared from the vineyard snail, binds specifically to carbohydrate molecules containing N-acetyl-D-galactosamine and has been shown to interact with neuraminidase treated human T lymphocytes (Hammarström, Hellström, Perlmann and Dillner, 1973). The property can be exploited to purify peripheral blood T lymphocytes using affinity chromatography (Hellström et al, 1976).

A commercial preparation of Helix pomatia lectin - Sepharose 6MB was used in all separations. The swollen beads are of a large size (macrobeads) and have a narrow size range (200 - 300μm) to ensure minimal trapping of lymphocytes during separation.

Reagents and Method

(i) PBS - HSA - NaN_3

A solution containing 0.2% human serum albumin (HSA) and 0.02% sodium azide (NaN_3) in PBS

(ii) PBS - HSA - NaN_3 (as above) + 0.1 mg/ml N-acetyl-α-D-galactosamine (NADG)

(iii) PBS - HSA - NaN_3 (as above) + 1.0 mg/ml NADG

(iv) Tris buffered HBSS (TBH)

(v) Neuraminidase

Approximately 3 ml of gel were transferred to a K9 column, fitted with a 80μm net, and eluted with 50 ml of PBS - HSA - NaN_3 buffer at a flow rate of 8 ml/min to remove merthiolate (preservative).

Washed mononuclear cells, prepared from 30 ml peripheral blood, were suspended in 2 ml TBH containing neuraminidase at a concentration of 5μg/ml. The cell suspension was incubated at 37°C for 45 minutes to expose lectin receptors, before washing twice with PBS - HSA - NaN_3.

The lymphocytes were counted, resuspended in 1.0 - 1.5 ml of PBS - HSA - NaN_3, applied to the column and allowed to penetrate the bed. The flow was then stopped and the cell suspension incubated on the gel at ambient temperature for 15 minutes. Unbound cells were then removed by eluting with 50 ml PBS - HSA - NaN_3 (flow rate

8 ml/min). Weakly and strongly bound cells were eluted using PBS - HSA - NaN_3 buffers containing 0.1 mg/ml and 1.0 mg/ml NADG respectively. The three populations of cells were washed and counted. E, EA and Facb rosettes were prepared on unfractionated and column fractionated populations.

SURFACE MEMBRANE IMMUNOGLOBULIN (sIg)

Immunofluorescence techniques were employed for the enumeration of sIg-positive mononuclear cells. These were performed by Mr. K. Case (Immunology Department, Royal United Hospital, Bath) using affinity column-purified fluoresceinated rabbit antibody against human Fd (μ) fragment.

Briefly, lymphocytes were isolated on Ficoll-Paque, incubated at 37°C for one hour and washed twice in warm (37°C) medium to remove surface labile IgG. The mononuclear cells were adjusted to 4×10^6/ml and to 1 ml of cells was added 1 drop of the antiserum. Following incubation at 4°C for 20 minutes, the cells were washed twice at 4°C and resuspended in 95% glycerol/5% PBS. Wet mounts were prepared and sIg-positive cells were enumerated using a Nikon fluophot microscope fitted with epifluorescence.

ESTERASE STAINING

Monocytes were enumerated using cell smears stained for α-naphthyl acetyl (non-specific) esterase (Lake, 1971). These were performed by Mr. S. Scott (Haematology Department, Royal United Hospital, Bath).

(vi) LYMPHOCYTE ACTIVATION BY MITOGENS

Three mitogens were used for lymphocyte transformation in vitro :-

Concanavalin A (Con A)

Phytohaemagglutinin (PHA)

Pokeweed Mitogen (PWM)

Con A was prepared immediately before use and filter sterilised (0.22μm filter; Millipore); PHA (400μg/ml) was prepared and stored in aliquots in a liquid nitrogen refrigerator and PWM was stored in aliquots at -20^{o}C.

Isolated, washed mononuclear cells were prepared in CMFSS and resuspended in lymphocyte medium (see Section I) to a concentration of 5 x 10^5/ml. Cell cultures were set up in quadruplicate using Nunc round-bottomed tissue culture plates (Gibco Bio Cult Ltd.). Each well contained :-

200μl mononuclear cells

10μl medium or mitogen solution

A range of mitogen concentrations was used and the optimum dosage determined. The plates were incubated for 65 - 70 hours in a humidified atmosphere of 95% air/5% CO_2.

Four hours before the cells were harvested (Titertek Cell Harvester; Flow Laboratories Ltd.), 0.5μCi of tritiated thymidine (specific activity 5 Ci/mmol) was added to each well. Radioactivity incorporated into trichloroacetic acid (TCA) - precipitable material was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 5574) using toluene/PPO/POPOP scintillation fluid.

The above method was used to assay the effect of Fc receptor positive cells on lymphocyte stimulation by mitogens. In these experiments only optimum mitogen concentrations were used. (See results Chapter 6).

In other experiments comparing Fc receptor expression before and after mitogen stimulation, bulk cultures were set up in sterile, plastic, flat bottomed tubes (Brunswick; Z/10/R). Again only optimum mitogen concentrations were used. EA and Facb rosettes were prepared on the activated and unstimulated lymphocytes.

(vii) FUNCTIONAL ASSAYS

NATURAL KILLER (NK) CELL ASSAY

A 16 hour assay was used based on the 4 hour assay described by Pape and co-workers (1979).

Facb and EA rosette depleted cell populations were prepared (Section II (iii)); sham depletions were also performed using unsensitised calf erythrocytes. Rosettes were set up on treated and untreated populations to confirm depletion and the remaining cells were adjusted to a concentration of 6×10^5/ml in K562 medium.

Chromium labelled (Cr^{51}) K562 cells were prepared by Dr. P. Bland (Clinical Investigation Department, Royal United Hospital, Bath) by incubation with 100μCi $^{51}CrO_4Na_2$ (specific activity 250-500μCi/μg) for 1 hour. The cells were washed x 3, diluted to 2×10^4/ml and the assays were set up in polypropylene screw top vials (Nunc;

Gibco Bio Cult Ltd.) to give a lymphocyte/target cell ratio of 60:1 :-

0.50 ml lymphocytes (ie 3×10^5 cells)

0.25 ml K562 (ie 5×10^3 cells)

Controls were prepared containing 0.5 ml medium or 0.5 ml unlabelled K562 cells at 6×10^5/ml in place of the lymphocyte suspension and the screw tops were loosely fitted. Whenever possible, assays were set up in quadruplicate, and the tubes maintained at $37^{\circ}C$ in a humidified atmosphere of 95% air/5% CO_2 for 16 hours.

After incubation the tops were screwed down and the tubes spun at 200 g for 10 minutes. A 0.5 ml aliquot of supernatant was withdrawn from each vial and expelled into an LP2 tube (Luckham). Pelleted cells were resuspended using a pasteur pipette and also expelled into an LP2 tube. The LP2 tubes were capped, placed into gamma counting vials (Gammavial B; Koch Light Laboratories Ltd.) and radioactivity monitored using the Packard Tri Carb liquid scintillation spectrometer.

Depleted and untreated cell populations were assessed in their ability to lyse K562 cells in the absence of antibody. Cytotoxicity was calculated as follows :-

Let a = number of counts in 0.5 ml supernatant

b = number of counts in 0.25 ml residue

Then total activity present = a + b

total activity in supernatant = a x 1.5

Therefore, % ^{51}Cr released = $\left\{\frac{a \times 1.5}{a + b}\right\} \times 100$

If S = % ^{51}Cr released by lymphocytes + K562

and K = % ^{51}Cr released by K562 alone

Then specific % ^{51}Cr released = S - K

KILLER (K) CELL ASSAY

Facb and EA rosette and sham (unsensitised calf erythrocytes) depleted cell populations were prepared and loss of rosetting cells confirmed as above.

A K cell assay using Chang liver cells, rabbit anti-Chang antiserum and the above prepared cell populations was carried out by Dr. P. Bland (Clinical Investigation, Royal United Hospital, Bath) according to the method of Panayi and Corrigall (1977).

Briefly, Chang liver cells (10^6) were radiolabelled with ^{51}Cr sodium chromate (100μCi for 1 hour at 37°C) and the washed labelled target cells, antiserum or medium and various lymphocyte populations were incubated together for 18 hours at 37°C in a total volume of 400μl. The assay tubes were then sedimented at 400 g for 10 minutes and 200μl of the supernatant was removed. Radioactivity present in the supernatants and pellets was determined.

Cultures comprised :-

100μl Chang (target cells)

100μl lymphocytes (effector cells) or medium or detergent

100μl rabbit anti-Chang serum (10^{-3} or 10^{-4} dilution) or medium

100μl medium

The following cultures were set up (all 400μl total volume) :-

1. targets + medium (spontaneous release)
2. targets + 100μl detergent + medium (maximum release)
3. targets + effectors + medium (SCMC)
4. targets + effectors + antiserum + medium (ADCC)

Calculation of Results

Let a = number of counts in 200μl supernatant

b = number of counts in 200μl residue

Then % ^{51}Cr released = $\left\{\frac{a \times 2}{a + b}\right\} \times 100$

If % ^{51}Cr released by cultures 1, 2, 3 and 4 is K, M, S and A respectively then

$$\underline{\text{Specific \% } ^{51}\text{Cr released (SCMC)}} = \frac{S - K}{M - K}$$

$$\underline{\text{Specific \% } ^{51}\text{Cr released (ADCC)}} = (A - K) - \left\{\frac{S - K}{M - K}\right\}$$

The mean maximum detergent release obtained is 90%

Spontaneous release is variable between 15% and 30%

CHAPTER THREE

ENUMERATION OF PERCENTAGE Fac_B ROSETTE FORMING CELLS IN HEALTHY SUBJECTS AND IN PATIENTS WITH RHEUMATIC DISEASES

SECTION I

(i) INTRODUCTION

It is generally accepted that RA has an immune-mediated pathology since large numbers of immunocompetent cells are detectable in rheumatoid synovia (Zvaifler, 1973) and the removal of lymphocytes by thoracic duct drainage leads to significant clinical improvement (Paulus et al, 1977). A major feature of RA is the production of both IgM and IgG rheumatoid factors (antiglobulins). This may imply an abnormal interaction between rheumatoid leucocytes and IgG and many authors have demonstrated an abnormal response of rheumatoid leucocytes to autologous, heat aggregated and complexed IgG (Eibl and Sitko, 1975; Weisbart, Bluestone and Goldberg, 1975; Hall, 1978). Raised numbers of EA-rosette forming cells in patients with RA have been reported (Bach, Delrieu and Delbarre, 1970; Scherak et al, 1976; Sharpin and Wilson, 1977) and Wooley and Panayi (1978) have detected abnormal binding of heat aggregated IgG by rheumatoid lymphocytes.

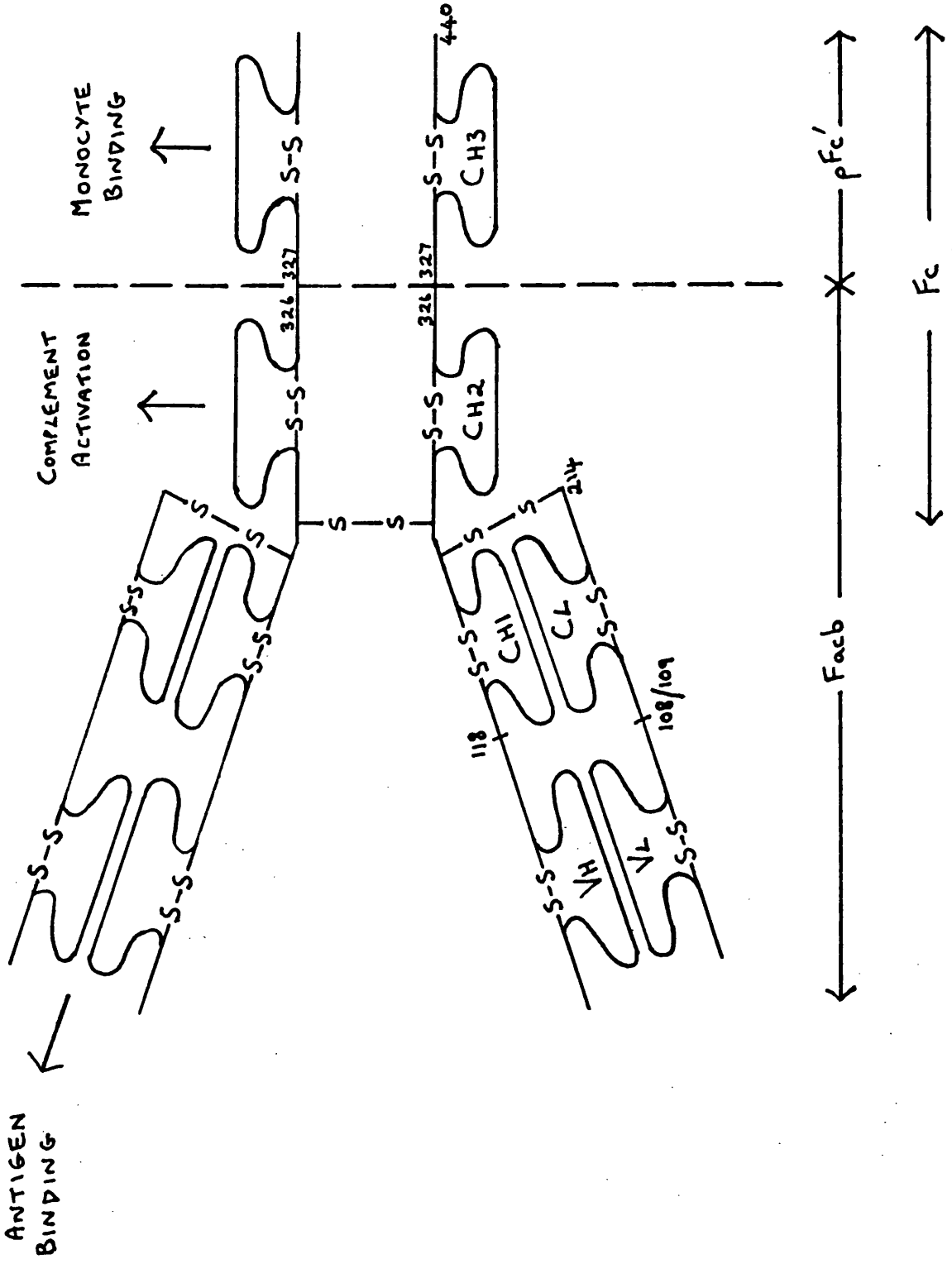
The Fc region of immunoglobulin consists of both the CH2 and CH3 domains (Fig. 3.1). Hall (1978) described specific migration inhibition of rheumatoid leucocytes by the Facb fragment of rabbit IgG. In order to examine possible differential binding by rheumatoid leucocytes, a rosette assay was developed using Facb sensitised calf erythrocytes (Chapter two, section iii) to measure Fc receptor binding by the CH2 region of IgG. Cells which bind the CH2 region of immunoglobulin will be termed Facb rosette forming cells. In some instances, E rosette and EA rosette forming cells were enumerated (Chapter two, section iii).

Fig. 3.1

SCHEMATIC REPRESENTATION OF IMMUNOGLOBULIN G

The basic structure of a molecule is shown opposite. Each loop in the heavy or light chain formed by an intrachain disulphide bond represents a single domain. There are one variable and three constant region domains. The Fc region encompasses both the second and third domains of the constant region of the immunoglobulin heavy chain, that is the CH2 and CH3 domains.

The heavy chain constant region of human IgG begins at the amino acid 118 while kappa light chain constant regions begin at 108 and the lambda light chain constant region at 109. These residue numbers apply to a human myeloma IgG1 protein.


ANTIGEN BINDING
COMPLEMENT ACTIVATION
MONOCYTE BINDING
S-S
S
326
327
440
CH1
CH2
CH3
CL
VH
VL
118
214
108/109
Fab
pFc'
Fc

SECTION II

Fc_{γ}-Receptor Bearing Cells in Health and Rheumatic Disease

Rabbit IgG and Facb fragments were prepared from anti-calf erythrocyte serum (Chapter two, section i). Calf erythrocytes were sensitised with various dilutions of the antibody to determine the maximum subagglutinating titre (Chapter two, section iii). Results obtained with one particular serum sample are presented in figs. 3.2 and 3.3. Each new preparation of IgG or Facb was titrated in this manner.

Patients with RA used in this study were receiving only analgesic or non-steroidal anti-inflammatory drug therapy. Control subjects consisted of healthy hospital personnel. Rheumatoid patients showed increased percentages of EA rosette forming cells at all the dilutions tested (Fig. 3.2). This difference was highly significant at all IgG titres between 1/400 and 1/6400. The 1/200 dilution was the maximum subagglutinating titre. Although percentage total EA rosettes were increased in RA, this increase was not significant at the 1/200 dilution. Increased percentage Facb rosette formation was also seen in RA patients (Fig. 3.3). This difference was highly significant at the 1/128 dilution ($p < 0.001$) and percentage rosette numbers detected using this dilution did not differ significantly from those detected using the 1/64 dilution, that is the maximum subagglutinating titre. A comparison of figures 3.2 and 3.3 shows that the Facb rosette forming cells do not account for all the $Fc_{\gamma}R$ positive cells but are a smaller population. It was found that there was a good correlation ($r = 0.784$) between percentage Facb rosettes and percentage EA rosettes at a dilution of 1/1600 (Fig. 3.4). Cells

Fig. 3.2

EA ROSETTE FORMATION BY RHEUMATOID AND HEALTHY MONONUCLEAR CELLS

Rosettes were prepared using various dilutions of IgG to sensitise calf erythrocytes. The maximum subagglutinating concentration is 1/200 but at this concentration rheumatoid and healthy subjects are not significantly different with respect to EA rosette percentages.

The columns and bars represent the mean $\pm$ 1 standard deviation.

The numbers of subjects tested are listed below.

$(\text{IgG Titre})^{-1}$	RA	HEALTHY	Student's t test
200	8	8	NS
400	10	10	$p < 0.02$
800	10	10	$p < 0.005$
1600	34	20	$p < 0.001$
3200	26	19	$p < 0.001$
6400	7	7	NS

NS = not significant

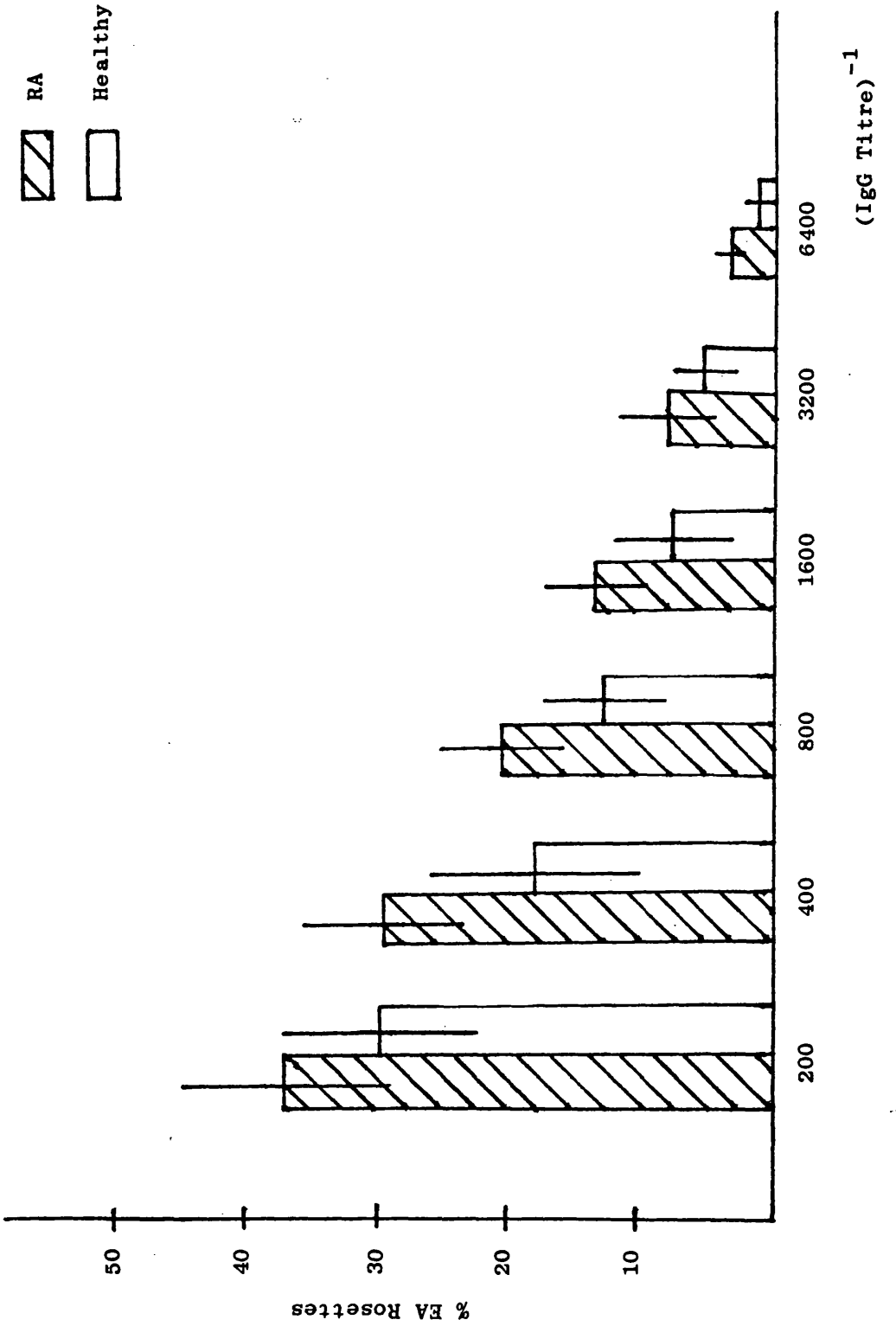

RA
Healthy
% EA Rosettes
50
40
30
20
10
200
400
800
1600
3200
6400
(IgG Titre)$^{-1}$

Fig. 3.3

Facb ROSETTE FORMATION BY RHEUMATOID AND HEALTHY MONONUCLEAR CELLS

Rosettes were prepared using various dilutions of Facb to sensitise calf erythrocytes. A dilution of 1/128 was chosen as the maximum subagglutinating concentration since the difference between rheumatoid and healthy subjects is highly significant and the values obtained do not differ significantly from the 1/64 dilution.

The columns and bars represent the mean $\pm$ 1 standard deviation.

The numbers of subjects tested are listed below.

(Facb Titre)$^{-1}$	RA	HEALTHY	Student's t test
64	4	4	$p < 0.02$
100	6	6	NS
128	27	18	$p < 0.001$
200	9	6	NS
400	3	2	NS

NS = not significant

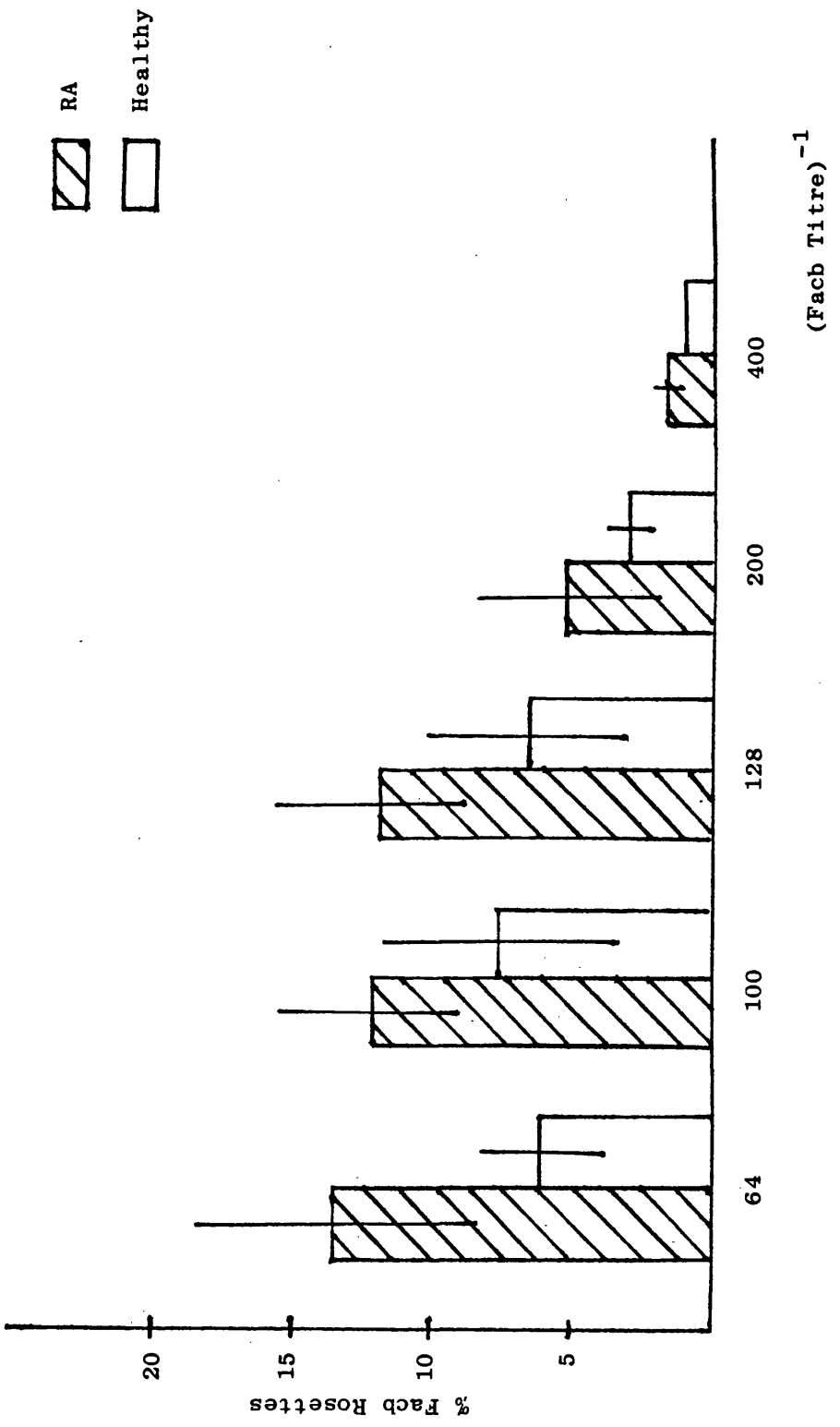

RA
Healthy
% Facb Rosettes
20
15
10
5
64
100
128
200
400
(Facb Titre)$^{-1}$

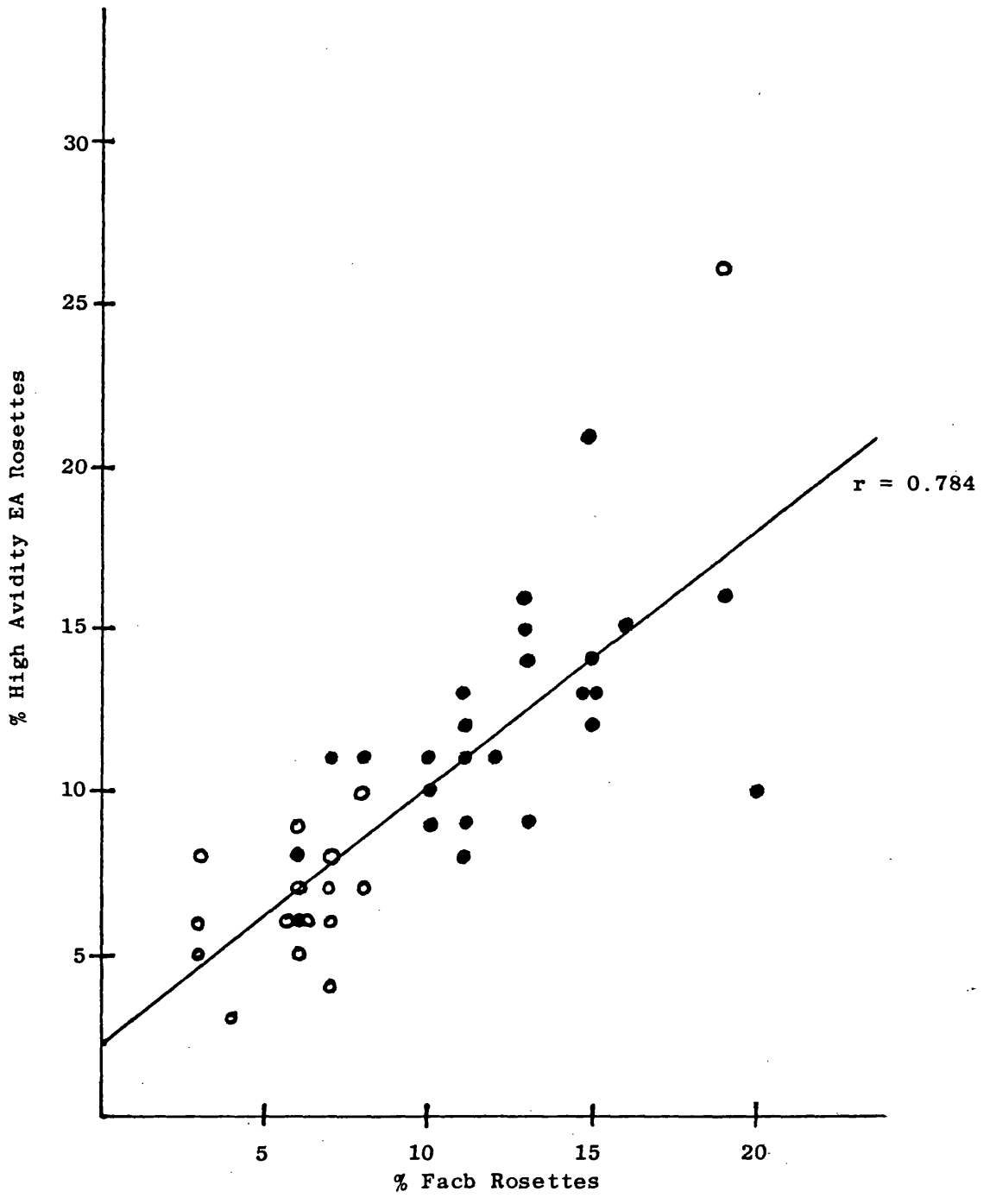
Fig. 3.4

CORRELATION BETWEEN HIGH AVIDITY EA ROSETTES (%) AND Facb ROSETTES (%)

Linear regression analysis was performed on the individual rosette values obtained for samples of mononuclear cells from healthy and rheumatoid donors. These values apply to IgG at 1/1600 and Facb at 1/128 (see Figs. 3.2 and 3.3).

This data fits the straight line:-

$$y = 0.789x + 2.345 \qquad (r = 0.784)$$


RA
Healthy
% High Avidity EA Rosettes
30
25
20
15
10
5
r = 0.784
5
10
15
20
% Facb Rosettes

forming EA rosettes at suboptimal titres will be referred to, in this thesis, as high avidity EA rosette forming cells.

In order to establish whether raised percentage Facb rosetting cells are unique to RA, peripheral blood lymphocytes were prepared from patients with other rheumatic diseases, namely osteoarthritis (OA) and ankylosing spondylitis (AS); percentage numbers of Facb rosette forming cells were scored. The results are shown in figure 3.5. Only patients with RA had significantly raised percentages of lymphocytes with Facb receptors compared to healthy controls ($p < 0.001$) although occasional samples with increased percentage Facb rosettes were seen in all groups. Absolute counts were not performed. It was noted that patients with AS who had raised percentage Facb rosettes often had peripheral joint involvement. This observation prompted attempts to correlate disease activity with the incidence of raised percentage Facb rosettes in patients with RA. No correlation was found. Sero-positive and sero-negative patients had similar percentage numbers of Facb rosettes and both groups showed significantly raised percentage Facb rosettes (figure 3.6; $p < 0.001$). Neither IgMRF nor IgGRF titres were related to percentage Facb rosettes (figure 3.7; IgGRF values were determined by Miss C. Allen, Bristol University). Plasma viscosity and erythrocyte sedimentation rate (ESR), often used as parameters of disease activity, showed no correlation with percentage Facb rosettes (figure 3.8). Patients assessed clinically as having active synovitis did not have enhanced percentage levels of Facb rosette forming cells (figure 3.9b) and synovial inflammation measured by quantitative thermography also showed no correlation with raised percentage of Facb rosettes (figure 3.10). A mean thermographic index of two or

Fig. 3.5

Facb ROSETTE FORMATION BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH RHEUMATOID ARTHRITIS (RA), OSTEOARTHRITIS (OA), ANKYLOSING SPONDYLITIS (AS) AND FROM HEALTHY CONTROLS

SUBJECTS	NO. TESTED	% Facb ROSETTES ($\pm$ 1 S.D.)	SIGNIFICANCE (Student's t test)
Healthy	18	6.6 ± 3.5	-
RA	27	12.2 ± 3.4	$p < 0.001$
OA	18	5.9 ± 3.5	NS
AS	24	8.7 ± 3.8	NS

NS = not significant

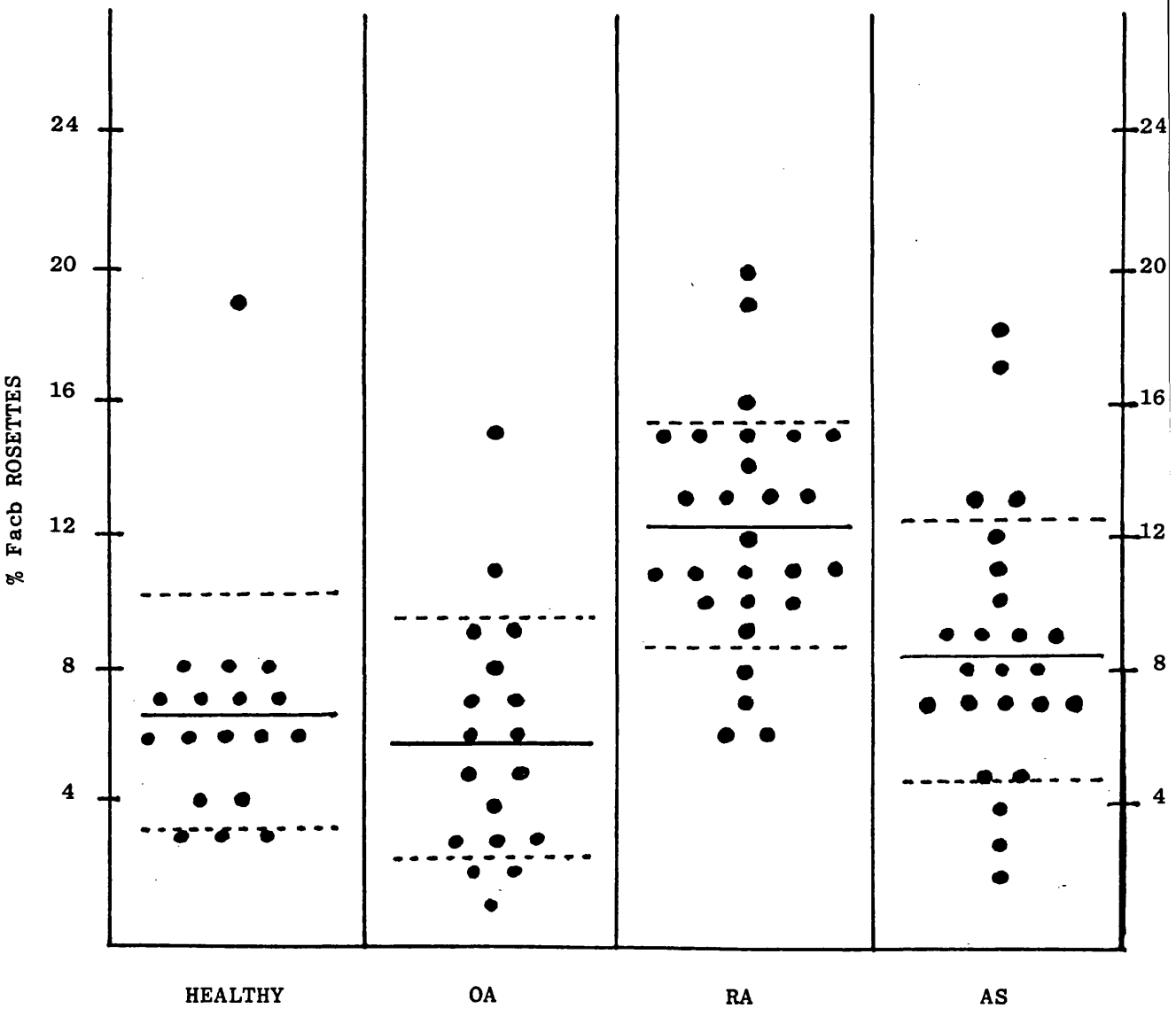

% Facb ROSETTES
24
20
16
12
8
4
HEALTHY
OA
RA
AS

Fig. 3.6

Facb ROSETTE FORMATION BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS WITH SERONEGATIVE AND SEROPOSITIVE RHEUMATOID ARTHRITIS (RA)

SUBJECTS	NO. TESTED	% Facb ROSETTES ($\pm$ 1 S.D.)
Seropositive RA	14	11.6 ± 2.8
Seronegative RA	10	12.9 ± 4.5
Healthy	18	6.6 ± 3.5

There is no significant difference between the two patient groups (Student's t test). Both seropositive and seronegative rheumatoid patients had raised numbers of percentage Facb rosette forming cells ($p < 0.001$).

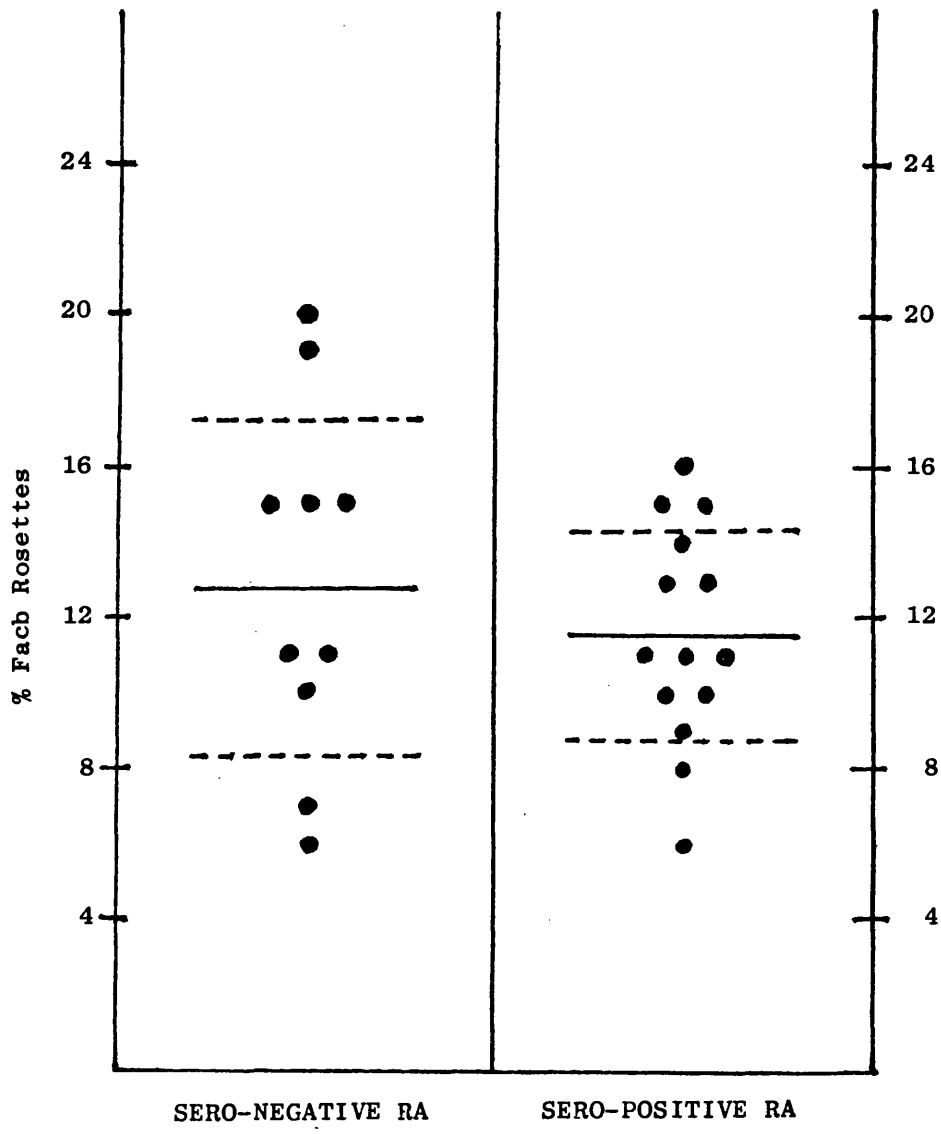
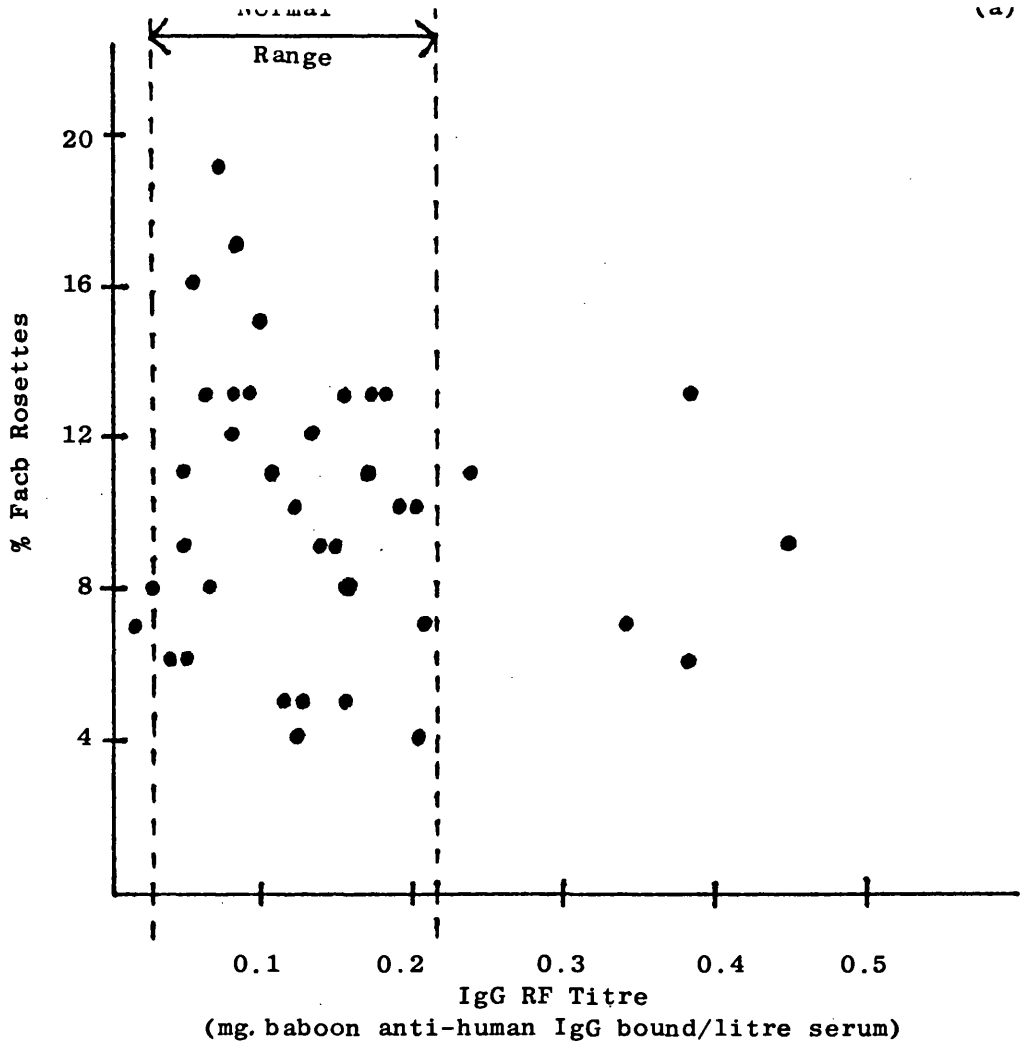

% Facb Rosettes
24
20
16
12
8
4
SERO-NEGATIVE RA
SERO-POSITIVE RA

Fig. 3.7

COMPARISON OF Facb ROSETTE FORMATION WITH ANTIGLOBULIN TITRES IN RHEUMATOID ARTHRITIS

Fig. 3.7 (a) shows IgG rheumatoid factor titres (IgGRF) in 38 patients with RA. There is no linear correlation with percentage Facb rosetting peripheral blood mononuclear cells. Only a small number of patients had raised levels of IgGRF while many patients had raised Facb rosettes. The IgGRF assay is described elsewhere (Allen, Elson, Scott, Bacon and Bucknall, 1981).

Fig. 3.7 (b) demonstrates a lack of linear correlation between percentage Facb rosettes and the differential agglutination titre in patients with RA. Many patients with raised levels of Facb rosettes were seronegative. Figs. 3.7 (b) and 3.6 represent the same patient group.


(a)
Normal
Range
20
16
12
8
4
% Facb Rosettes
0.1
0.2
0.3
0.4
0.5
IgG RF Titre
(mg. baboon anti-human IgG bound/litre serum)

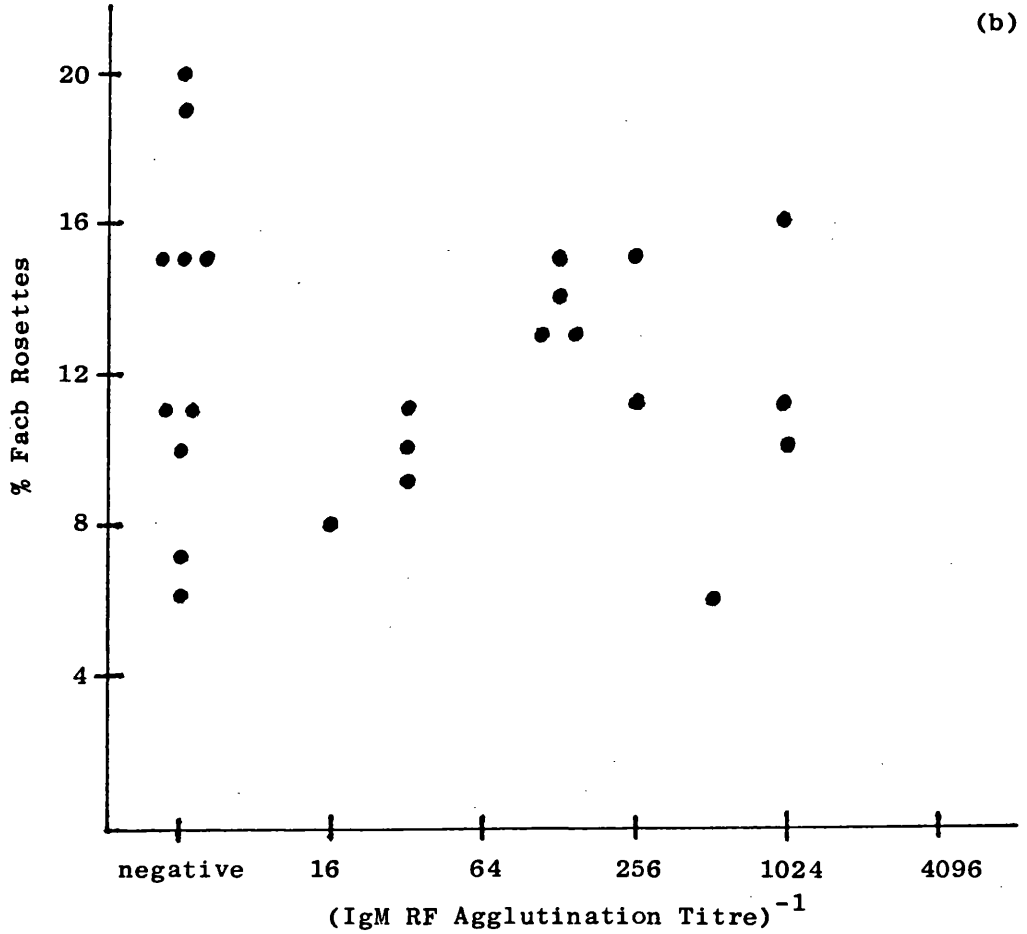

(b)
20
16
12
8
4
% Facb Rosettes
negative
16
64
256
1024
4096
(IgM RF Agglutination Titre)$^{-1}$

Fig. 3.8

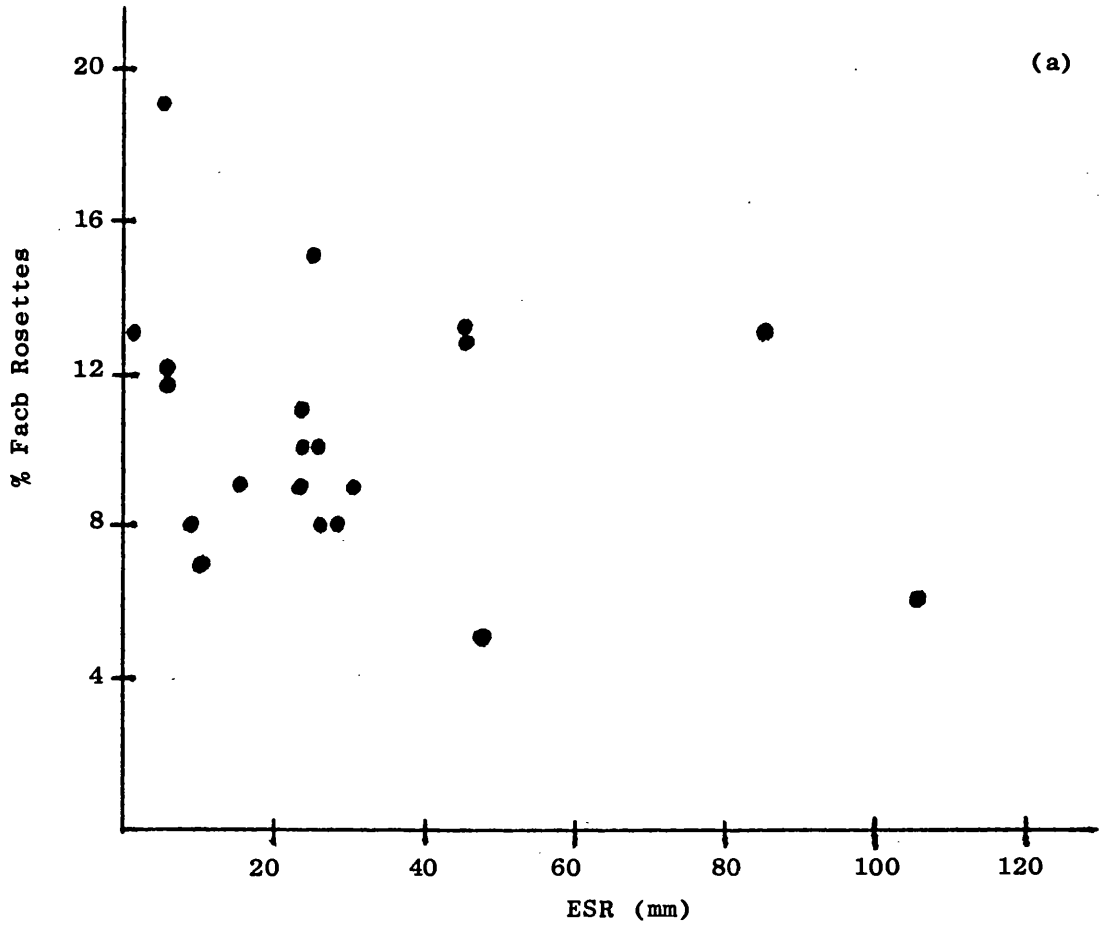
COMPARISON OF Facb ROSETTE FORMATION WITH

ERYTHROCYTE SEDIMENTATION RATE (ESR) AND

PLASMA VISCOSITY (pV) IN RHEUMATOID ARTHRITIS

Figs. 3.8 (a) and 3.8 (b) demonstrate a lack of linear correlation between percentage Facb rosettes and ESR or pV in rheumatoid patients.

(a)


20
16
12
8
4
% Facb Rosettes
20
40
60
80
100
120
ESR (mm)

(b)

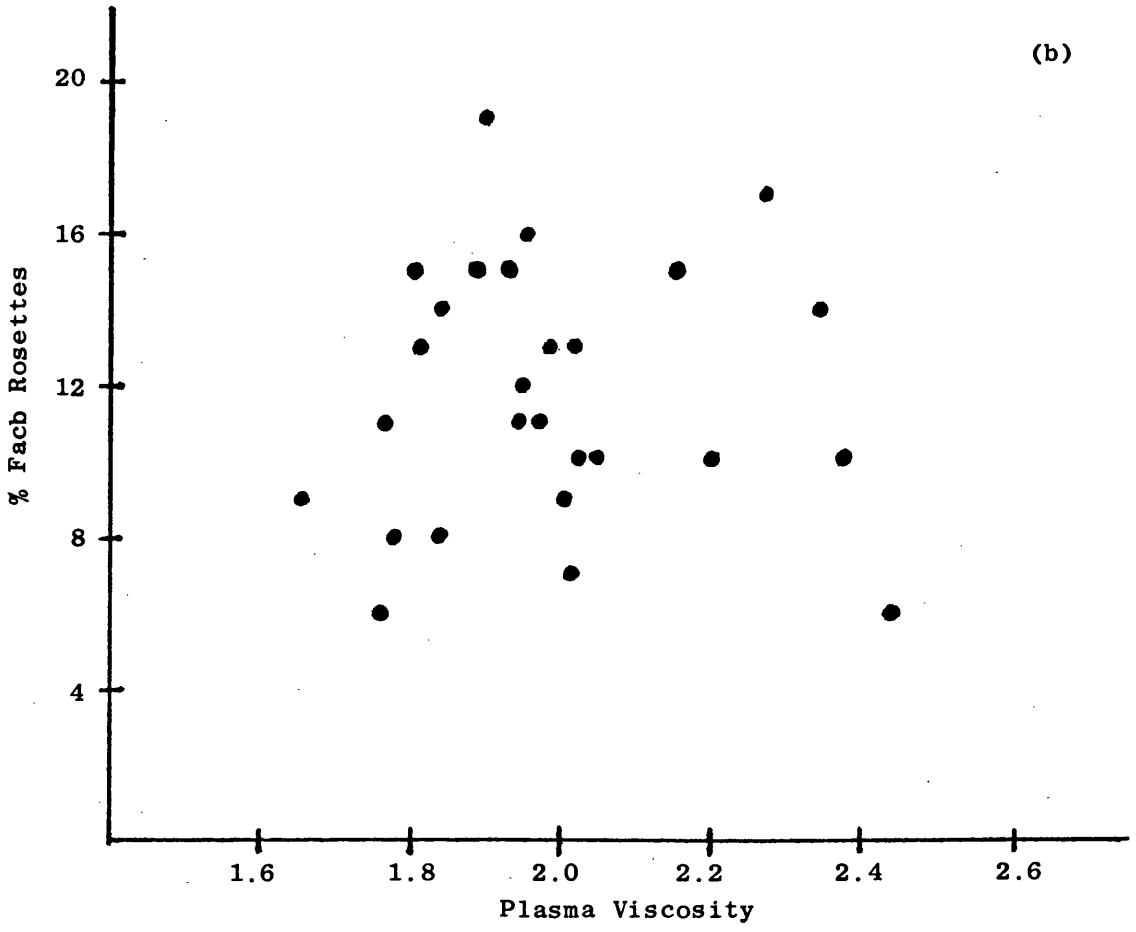

20
16
12
8
4
% Facb Rosettes
1.6
1.8
2.0
2.2
2.4
2.6
Plasma Viscosity

Fig. 3.9

Facb ROSETTE FORMATION BY RHEUMATOID PERIPHERAL BLOOD MONONUCLEAR CELLS. COMPARISON WITH DISEASE DURATION AND ACTIVE SYNOVITIS

Fig. 3.9 (a)

Facb rosettes show raised levels irrespective of disease duration. However, patients with early RA (i.e. two years' duration) possessed very high levels of Facb rosetting cells. All patients were receiving non-steroidal anti-inflammatory therapy.

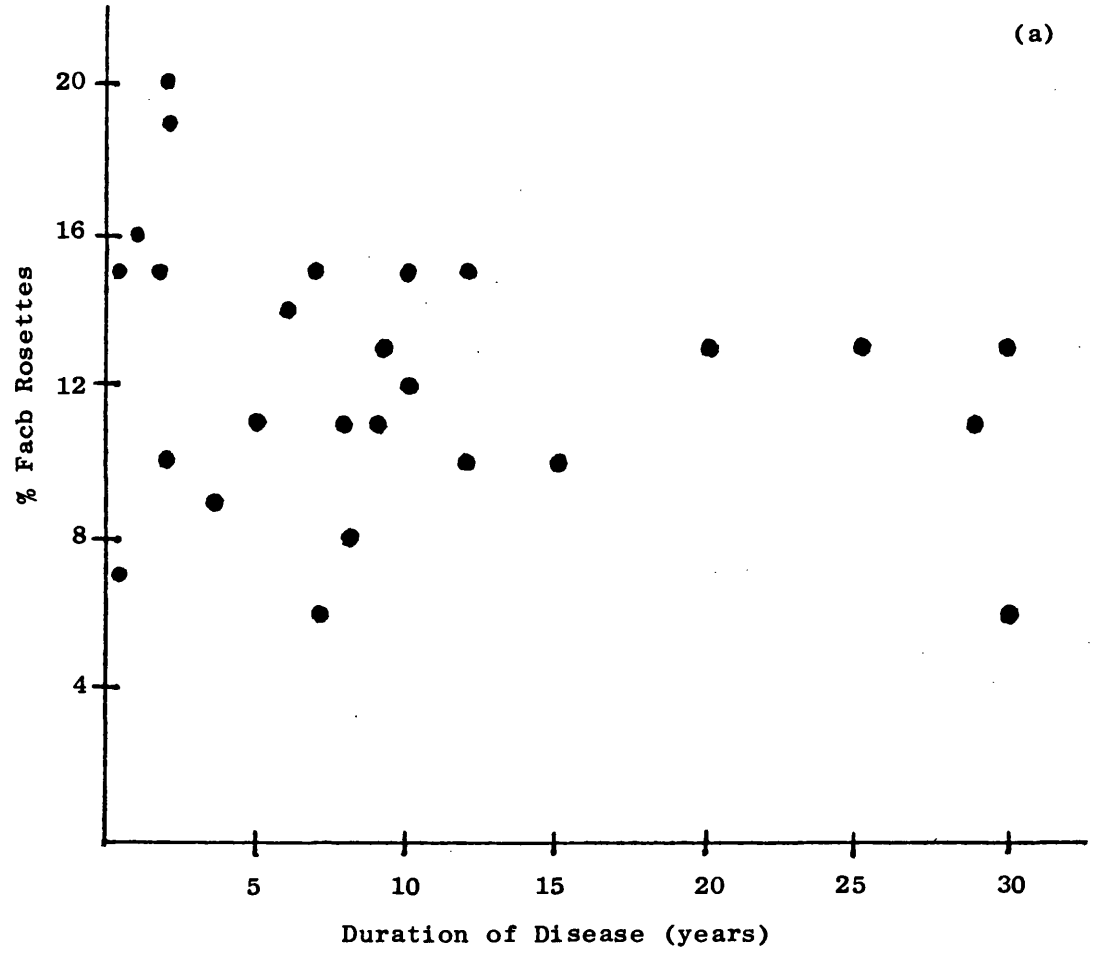
SUBJECTS	NO. TESTED	% Facb ROSETTES ($\pm$ 1 S.D.)
Early RA ($\leq$ 2 yrs)	7	15 $\pm$ 4
RA ($>$ 2 yrs)	19	11 $\pm$ 3

The above difference is significant ($p < 0.02$) using the Student's t test.

Fig. 3.9 (b)

Patients assessed as having active synovitis did not have significantly raised levels of percentage Facb rosettes.

SUBJECTS	NO. TESTED	% Facb ROSETTES ($\pm$ 1 S.D.)
Active RA	9	13 $\pm$ 3
Inactive RA	10	12 $\pm$ 4


(a)
20
16
12
8
4
% Facb Rosettes
5
10
15
20
25
30
Duration of Disease (years)

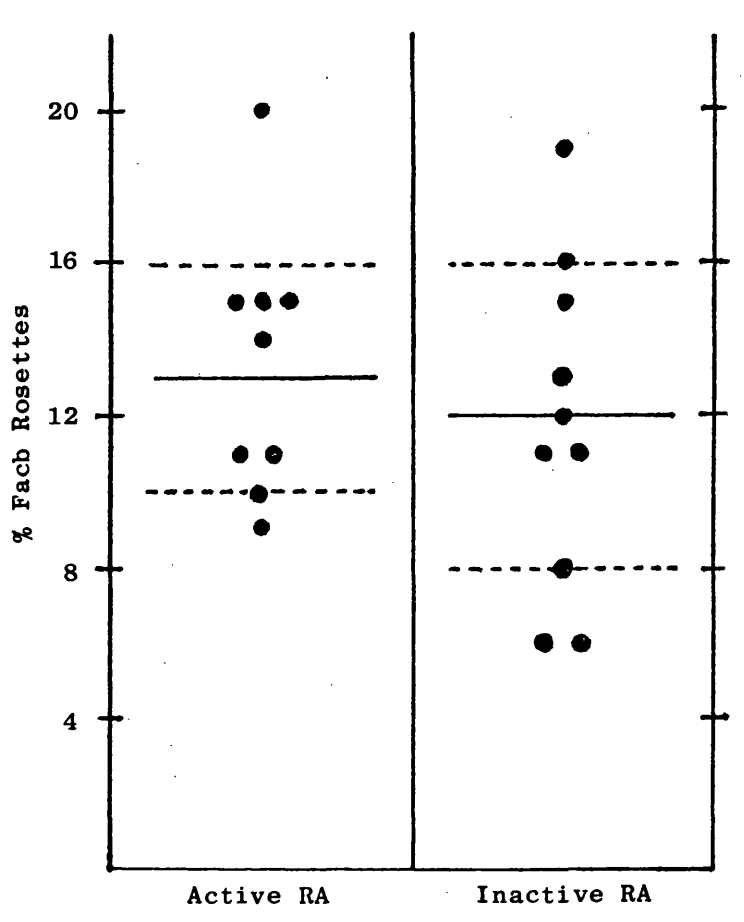

(b)
20
16
12
8
4
% Facb Rosettes
Active RA
Inactive RA

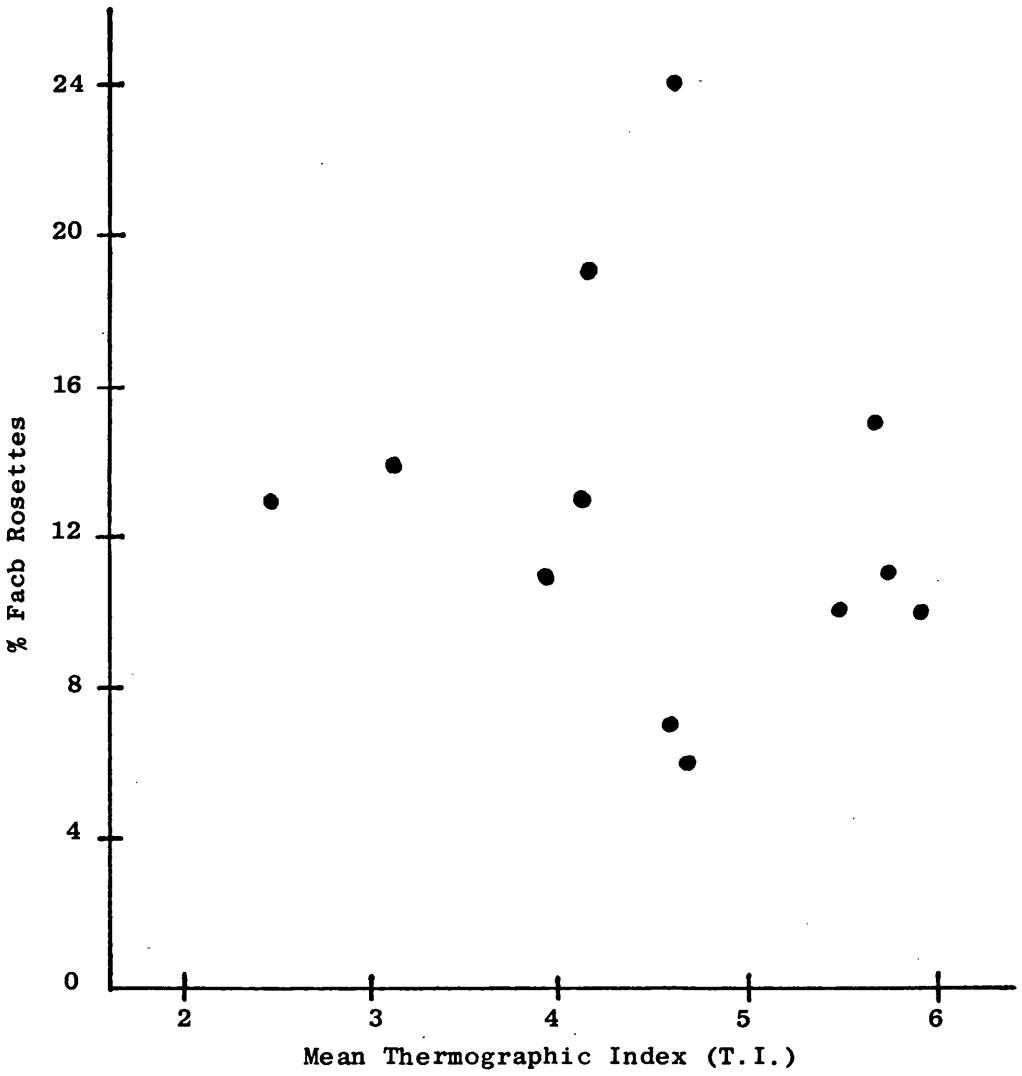
Fig. 3.10

Facb ROSETTE FORMATION IN RHEUMATOID ARTHRITIS

COMPARISON WITH SYNOVIAL INFLAMMATION MEASURED

BY QUANTITATIVE THERMOGRAPHY

The mean thermographic index of two or four joints was calculated (Table 3.1) and the results plotted against percentage Facb rosettes. There is no linear correlation between the results.


24
20
16
12
8
4
0
% Facb Rosettes
2
3
4
5
6
Mean Thermographic Index (T.I.)

four joints was obtained and compared with percentage peripheral blood Facb rosettes (Table 3.1). Although no correlation was shown to exist between percentage Facb rosettes and disease duration, it was noted that patients with early RA (i.e. 2 years or less) had significantly increased percentages of Facb rosettes ($p < 0.02$; Figure 3.9a). One patient (LK) followed over a period of 12 months from diagnosis showed a rapid rise in Facb rosette forming cells (Table 3.2). No other serial data was compiled during this work but such studies are, at present, being investigated. Another individual (LW) also presented at clinic with a high differential agglutination titre (DAT $>$ 1/4000) but no clinical symptoms. He also had low percentage Facb rosettes (4%). This individual did not develop RA.

A number of rheumatoid patients, members of a long-term study in progress at the RNHRD, were tissue typed. These patients were receiving a wide variety of drug treatment including steroidal and immunosuppressive therapy. Percentage peripheral blood Facb rosettes were evaluated. No correlation was found between percentage Facb rosettes and DR4 positivity (Figure 3.11). Both DR4 positive and DR4 negative rheumatoid patients had higher percentage Facb rosettes than controls ($p < 0.001$ and $p < 0.05$ respectively). However, the control group was not tissue typed.

The lack of correlation observed between synovitis, a major feature of early RA, and percentage Facb rosette forming cells (Figs. 3.9b and 3.10) may reflect the use of peripheral blood lymphocytes. The response may be localised to the major site of inflammation, that is the synovium. Paired peripheral blood and

TABLE 3.1

THERMOGRAPHIC INDICES OBTAINED FOR RHEUMATOID PATIENTS WITH SYNOVITIS

The mean thermographic index (T.I.) of two or four joints was calculated. Facb rosettes were prepared using peripheral blood mononuclear cells.

SUBJECT	THERMOGRAPHIC INDEX (T.I.)				MEAN T.I.	% Facb ROSETTES
	LEFT KNEE	RIGHT KNEE	LEFT HAND	RIGHT HAND		
RA 1	3.58	4.28	-	-	3.93	11
RA 2	2.75	2.52	3.72	3.53	3.13	14
RA 3	5.28	5.13	4.52	3.74	4.67	6
RA 4	6.05	5.80	5.69	6.05	5.90	10
RA 5	-	-	2.18	2.71	2.45	13
RA 6	5.23	6.14	6.04	5.55	5.74	11
RA 7	4.85	4.69	3.88	4.93	4.59	24
RA 8	5.97	6.33	2.58	3.50	4.60	7
RA 9	6.14	6.21	5.01	5.33	5.67	15
RA 10	5.25	5.89	5.69	5.12	5.48	10
RA 11	3.40	3.73	4.85	4.63	4.16	19
RA 12	3.64	4.77	4.20	3.85	4.12	13

TABLE 3.2

Facb ROSETTE FORMING PERIPHERAL BLOOD MONONUCLEAR CELLS. A SERIAL STUDY OF ONE INDIVIDUAL (L.K.)

DATE	COMMENTS	TOTAL EA ROSETTES	Facb ROSETTES
21/12/78	Minimal symptoms; DAT 1/1024	22%	4%
25/1/79	Diagnosed as early RA	17%	3%
4/5/79	Widespread peripheral joint involvement; treatment DF118 only	32%	22%
9/11/79	Non-steroidal anti-inflammatory therapy	-	22%

Fig. 3.11

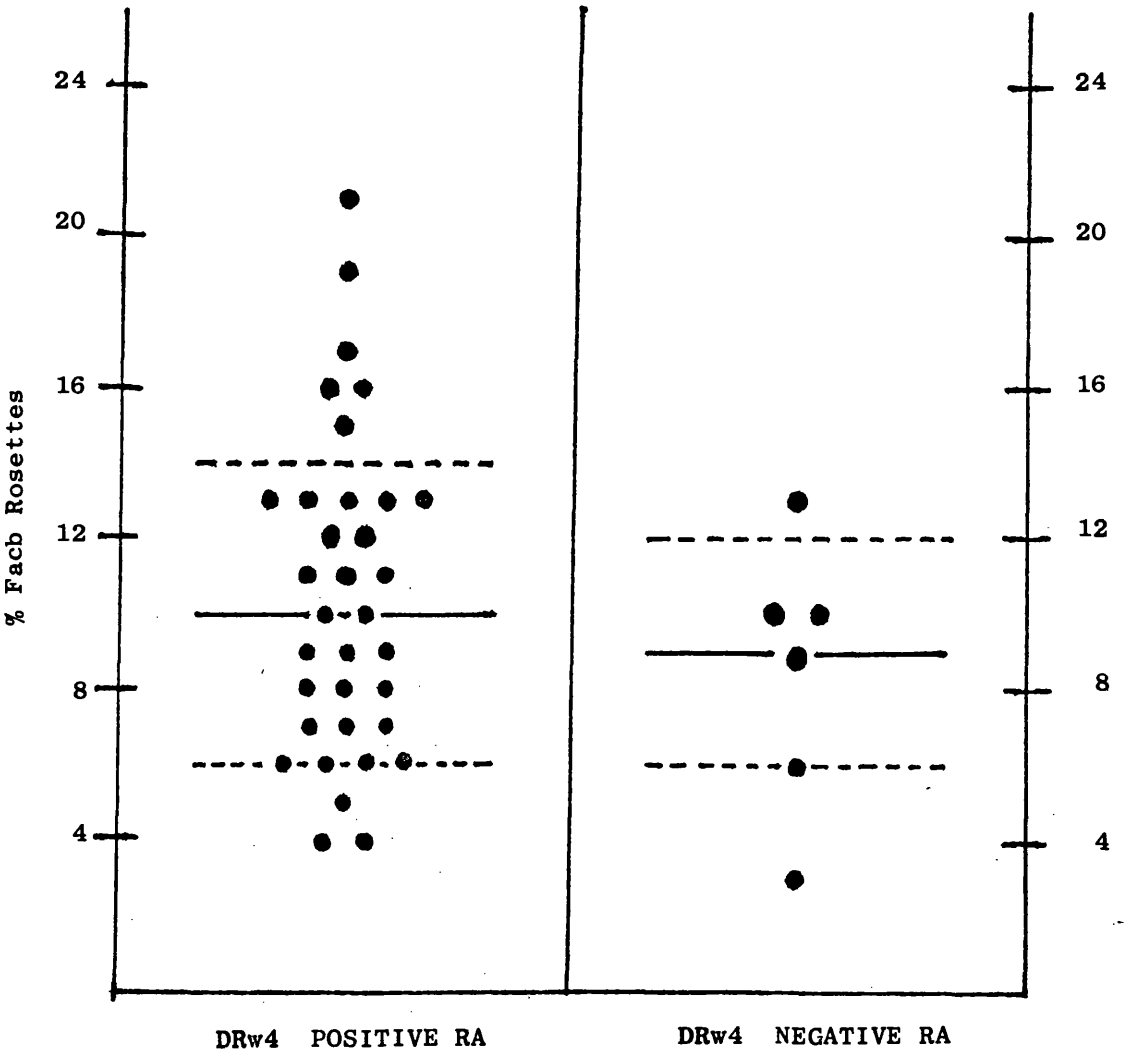
Facb ROSETTE FORMATION IN DRw4 POSITIVE AND DRw4 NEGATIVE

RHEUMATOID ARTHRITIS PATIENTS

SUBJECTS	NO. TESTED	% Facb ROSETTES ($\pm$ 1 S.D.)
DRw4 positive RA	34	10 ± 4
DRw4 negative RA	6	9 ± 3
Healthy (not shown)	16	5 ± 4

There is no significant difference in Facb rosette formation in DRw4 positive and DRw4 negative RA patients

Both DRw4 positive and DRw4 negative RA patients have significantly higher percentages of Facb rosette forming cells ($p < 0.001$ and $p < 0.05$ respectively) than healthy subjects.


% Facb Rosettes
24
20
16
12
8
4
DRw4 POSITIVE RA
DRw4 NEGATIVE RA

synovial fluid samples were obtained from rheumatoid patients with active synovitis on a variety of anti-inflammatory drugs. The results are expressed diagramatically in figure 3.12. Percentage total E, EA and Facb rosettes were enumerated. As previously stated, the term high avidity EA rosettes has been used to describe those cells which form EA rosettes under suboptimal conditions, that is with low concentrations of IgG coating the calf erythrocytes (figure 3.2; IgG titre of 1/1600). No strict measurements of receptor avidity were made. Mononuclear cells, harvested from a ficoll-hypaque gradient, were used for rosette formation but the preparation may contain other contaminating cell types of similar density. The distribution of rosette forming cells within rheumatoid peripheral blood and synovial fluid was virtually identical (figure 3.12). Only the percentage of high avidity EA rosettes was significantly different. Synovial fluid was found to contain lower percentage numbers ($p < 0.05$) of high avidity EA-rosette forming cells; this may be due to blocking of receptors by immune complexes in the joint fluid, which are not removed by washing. No parallel decrease of Facb rosette forming cells was observed. This may reflect differences in immune complex binding by Facb and high avidity EA rosette forming cells.

Alexander, Titus and Segal (1978) have reported that erroneous estimations of FcRs can be made using ficoll-hypaque prepared lymphocytes due to binding of cytophilic immunoglobulin induced by ficoll-hypaque, and pre-washing of whole blood is recommended. Experiments were carried out to determine whether pre-washing affected the relative percentages of Facb rosettes detected. It is possible that serum IgG is bound more avidly by control lymphocytes than by

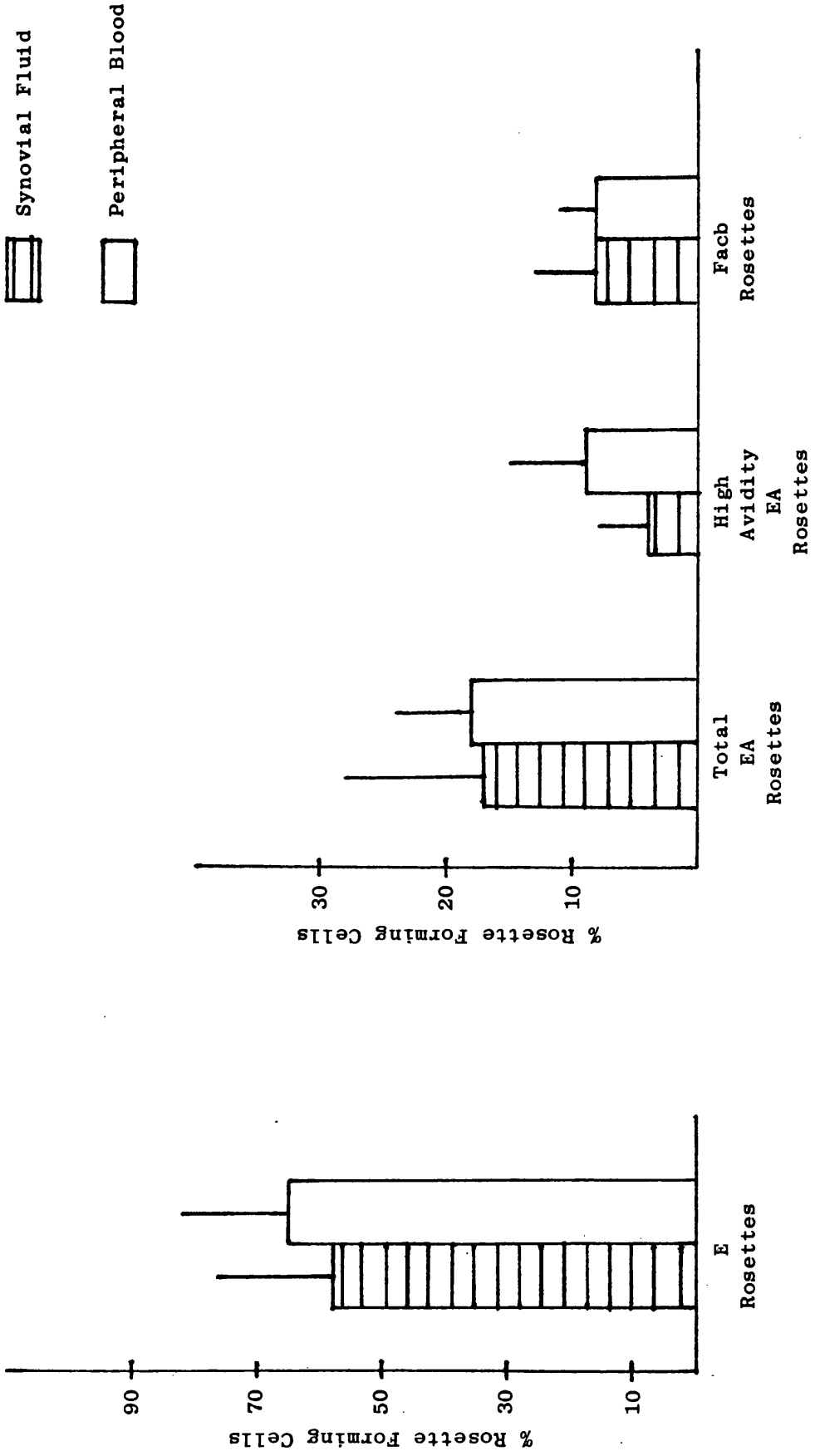
Fig. 3.12

ROSETTE FORMATION BY RHEUMATOID PERIPHERAL BLOOD AND SYNOVIAL FLUID MONONUCLEAR CELLS

Mononuclear cells were prepared from rheumatoid peripheral blood and synovial fluid using ficoll hypaque. Percentage E, EA and Facb rosettes were enumerated. The results are expressed as the mean $\pm$ 1 S.D. In some instances synovial fluid was obtained from two joints and rosettes assessed separately; in all these cases, similar rosette numbers were found in the two fluids.

	PERIPHERAL BLOOD	SYNOVIAL FLUID
E Rosettes	65 ± 17 (n = 4)	58 ± 18 (n = 7)
Total EA Rosettes	18 ± 6 (n = 10)	17 ± 11 (n = 15)
High Avidity EA Rosettes	9 ± 6 (n = 10)	4 ± 4 (n = 13)
Facb Rosettes	8 ± 3 (n= 10)	8 ± 5 (n = 15)

Only high avidity EA rosettes showed significant differences. Peripheral blood high avidity EA rosettes were significantly increased ($p < 0.05$)


Synovial Fluid
Peripheral Blood
% Rosette Forming Cells
10
30
50
70
90
E Rosettes
% Rosette Forming Cells
10
20
30
Total EA Rosettes
High Avidity EA Rosettes
Facb Rosettes

rheumatoid lymphocytes and the observed differences in percentage Facb rosettes may be lost. The results (figure 3.13) show that, in all cases, pre-washing of whole blood increased the percentage of detectable rosettes, but this washing only served to increase the observed differences between patients with RA and healthy subjects; only rheumatoid patients showed significantly increased percentage values of Facb and high avidity EA rosettes after washing. In this experiement, three of the five patients had low levels of Facb rosettes while two of the control subjects had very high levels resulting in a lack of significance between rheumatoid and healthy subjects with respect to percentage Facb rosette forming cells. This lack of significance was probably due to the small number of estimations compared. It was also noted that lymphocyte preparations from pre-washed blood contained increased numbers of contaminating polymorphonuclear cells and it may be advisable to repeat this experiment using purified lymphocyte populations (i.e. following carbonyl iron treatment).

S E C T I O N I I I

The Significance of Raised $Fc_{\gamma}R$ Bearing Cells

As previously stated, raised percentages of Facb rosettes were detectable in occasional samples of all the groups tested (figure 3.5). The percentage Facb rosettes detected in normal healthy individuals varied from day to day. Occasional raised values were seen; serial results for two female and two male individuals are shown in table 3.3. For example, subject VW showed a high value of 11% in March 1979; similarly DP had a raised value of 9% in

Fig. 3.13

EFFECT OF PRE-WASHING OF WHOLE BLOOD ON THE DETECTION OF LYMPHOCYTE SUBPOPULATIONS BY EA AND Facb ROSETTES

Heparinised venous peripheral blood (approx. 5 ml) was washed three times with copious amounts of CMFSS. Both washed and unwashed blood from healthy and rheumatoid donors were used for lymphocyte isolation. EA and Facb rosettes were prepared. Percentage rosette forming cells detected in washed and unwashed preparations were compared. The results are presented numerically and diagramatically. Washing significantly increased the number of rosettes detected in rheumatoid but not healthy peripheral blood.

SUBJECTS	PROCEDURE	TOTAL EA ROSETTES (%)	HIGH AVIDITY EA ROSETTES (%)	Facb ROSETTES (%)
RA	Washed	34 ± 13	11 ± 4 } *	11 ± 2 } **
(n = 5)	Unwashed	27 ± 15	4 ± 2 } *	6 ± 3 } **
Healthy	Washed	26 ± 8	10 ± 10	7 ± 5
(n = 5)	Unwashed	24 ± 11	9 ± 8	6 ± 3

Student's t test * $p < 0.01$

** $p < 0.05$

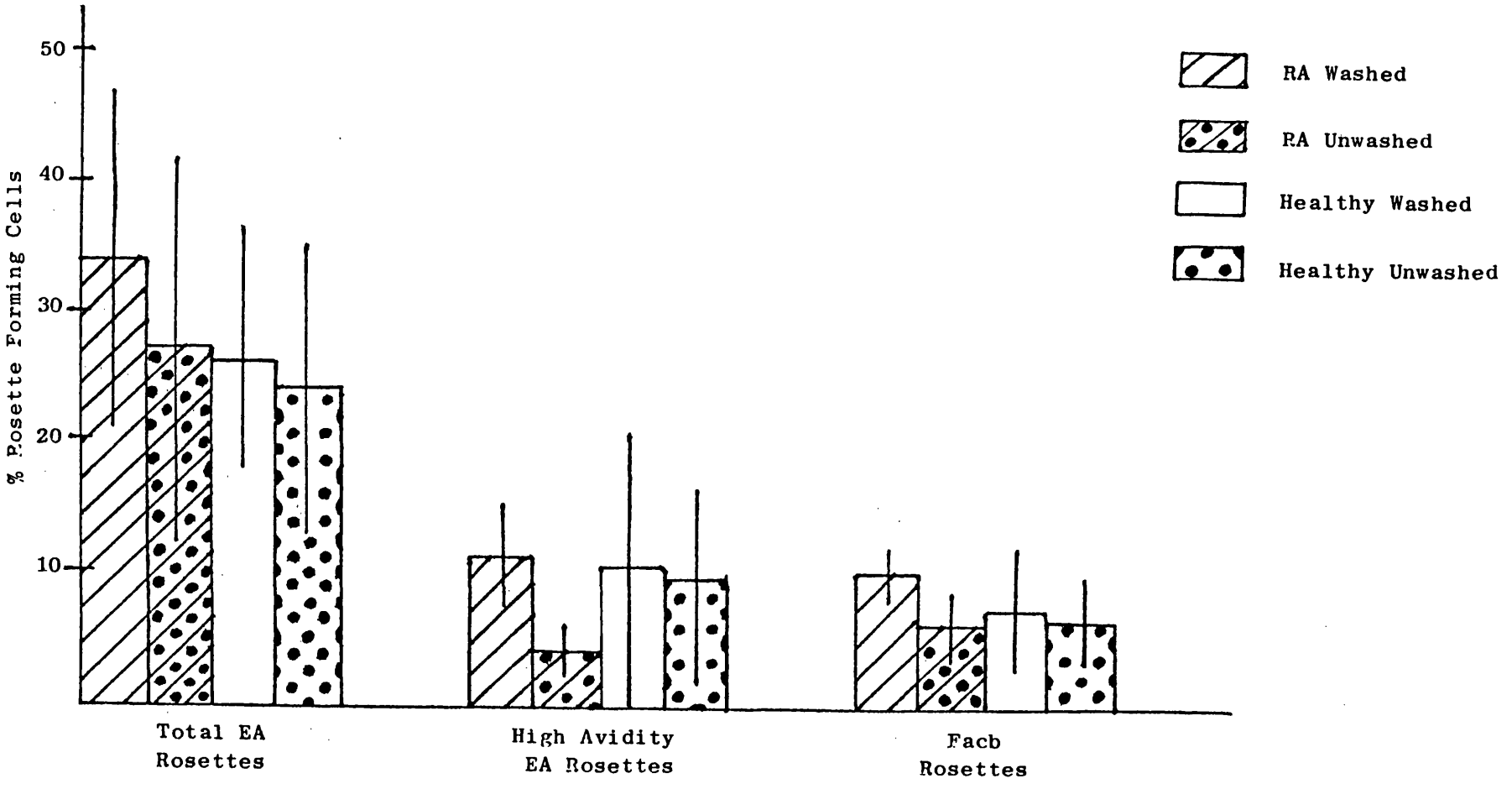

% Rosette Forming Cells
50
40
30
20
10
RA Washed
RA Unwashed
Healthy Washed
Healthy Unwashed
Total EA Rosettes
High Avidity EA Rosettes
Facb Rosettes

TABLE 3.3

Facb ROSETTE FORMATION BY PERIPHERAL BLOOD MONONUCLEAR CELLS OF HEALTHY INDIVIDUALS

SUBJECT	DATE	Facb ROSETTES	DATE	Facb ROSETTES	Mean ± 1 S.D.
VW (♀)	June 1978	8%	May 1979	1%	4 ± 3 (%)
	Aug. 1978	2%	Dec. 1979	2%	
	Sept. 1978	7%	June 1980	5%	
	Nov. 1978	1%	Aug. 1980	1%	
	Nov. 1978	3%	Sept. 1980	4%	
	Jan. 1979	4%	Oct. 1980	1%	
	Feb. 1979	1%	Dec. 1980	2%	
	Feb. 1979	3%	Dec. 1980	6%	
	March 1979	11%	Jan. 1981	8%	
	May 1979	1%	Jan. 1981	6%	
	May 1979	1%			
DP (♀)	Aug. 1978	5%			4 ± 3 (%)
	Oct. 1978	9%			
	June 1979	1%			
	Dec. 1979	3%			
	Jan. 1981	3%			
NH (♂)	July 1978	8%	May 1979	16%	11 ± 5 (%)
	Jan. 1979	3%	Oct. 1979	16%	
	March 1979	19%	Dec. 1979	6%	
	April 1979	13%	Nov. 1980	8%	
KB (♂)	Jan. 1979	2%			5 ± 3 (%)
	June 1980	7%			
	June 1980	7%			
	July 1980	2%			

October 1978 but this value is still within the normal range. Subject NH had high percentage levels of Facb rosettes on all occasions tested over a period of several months; this high level was not associated with one particular preparation of Facb. All the values obtained for KB were within the normal range. One individual, admitted to the RNHRD with orchitis due to mumps virus infection, also had a very high percentage of Facb rosette forming cells (17%; Table 3.4). The possibility that Facb rosette forming cells are raised in response to infection was investigated. Peripheral blood lymphocytes from rheumatoid patients with concurrent infections were prepared and percentage Facb rosettes enumerated. The values were not significantly raised when compared with uninfected rheumatoid patients (Table 3.4). A different approach was made. Mononuclear cells were prepared from healthy subjects having skin tests prior to possible BCG vaccination and from one subject receiving an influenza vaccine. The results are shown in Figs. 3.14 and 3.15. The PPD skin tests produced an increase of percentage Facb rosettes in sensitised individuals (Fig. 3.14). Inoculation with 'flu' vaccine also produced an increase in percentage Facb rosette forming cells (Fig. 3.15).

TABLE 3.4

Facb ROSETTE FORMATION BY PERIPHERAL BLOOD MONONUCLEAR CELLS. EFFECT OF INFECTION BY MUMPS VIRUS AND OTHER INFECTIVE AGENTS

SUBJECT	DIAGNOSIS	% Facb ROSETTES
PE	mumps/orchitis	17
SA	RA + pneumonia	13
PH	RA + pneumonia + myeloma + amyloid	14
JO	RA + septic severe chronic chest infection	10
HAR	septic arthritis	6
HAN	septic arthritis	7
		Mean % Facb ROSETTES $\pm$ 1 S.D. 10 ± 3

Figure 3.14

PERIPHERAL BLOOD Facb ROSETTE FORMING CELLS

EFFECTS OF SKIN TESTING

Three healthy individuals having PPD skin tests prior to possible Bacillus Calmette-Guerin (BCG) vaccination were used for study. Percentage peripheral blood Facb Rosettes were evaluated before skin testing and on days 3 and 5 following testing. The results are shown opposite. Two individuals showed positive results to the skin test while the third was skin test negative.

	% Facb ROSETTES			
SUBJECT	DAY 1	DAY 3	DAY 5	SKIN TEST RESULT
1	3	11	3	positive
2	7	11	4	positive
3	5	8	4	negative

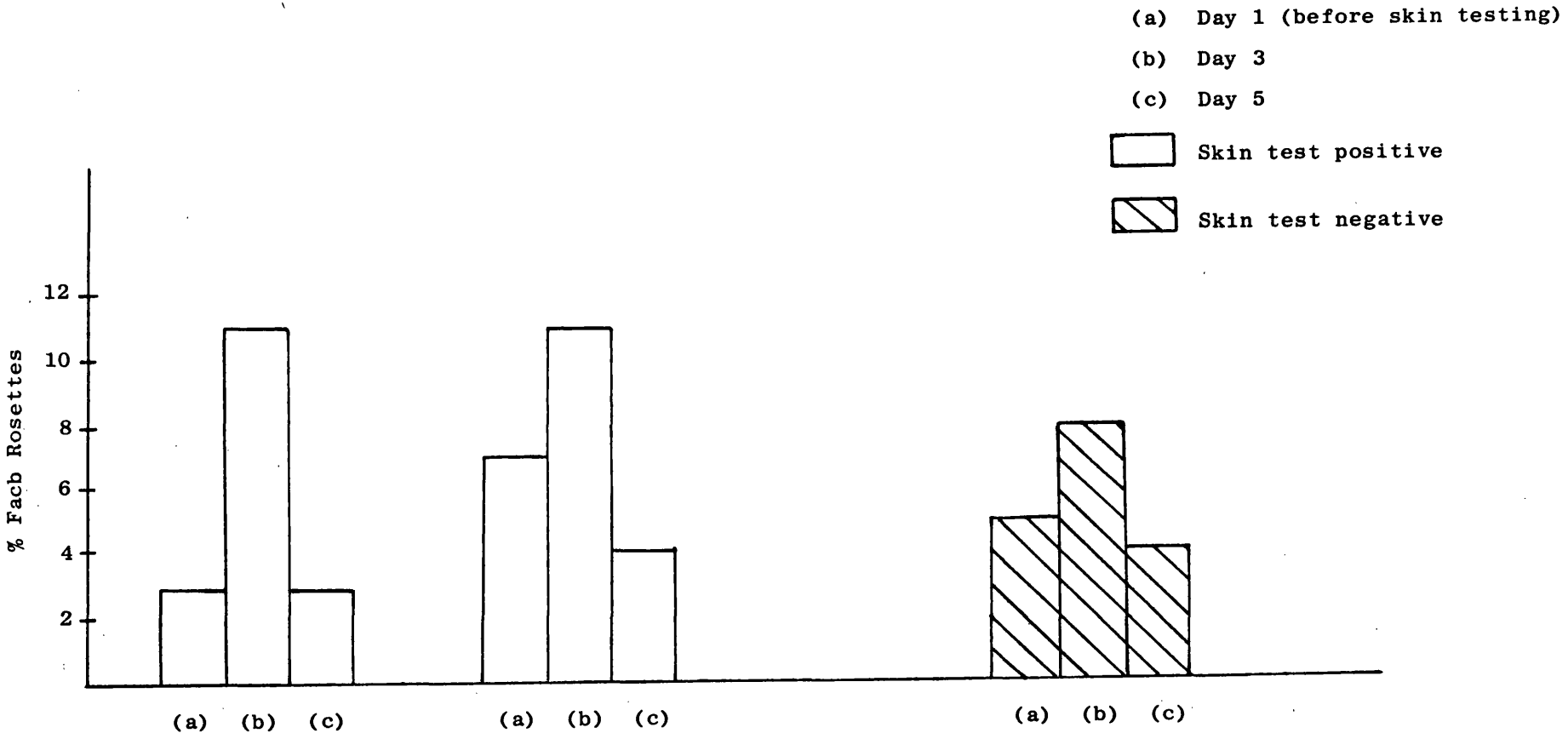

(a) Day 1 (before skin testing)
(b) Day 3
(c) Day 5
Skin test positive
Skin test negative
% Facb Rosettes
12
10
8
6
4
2
(a) (b) (c)
(a) (b) (c)
(a) (b) (c)

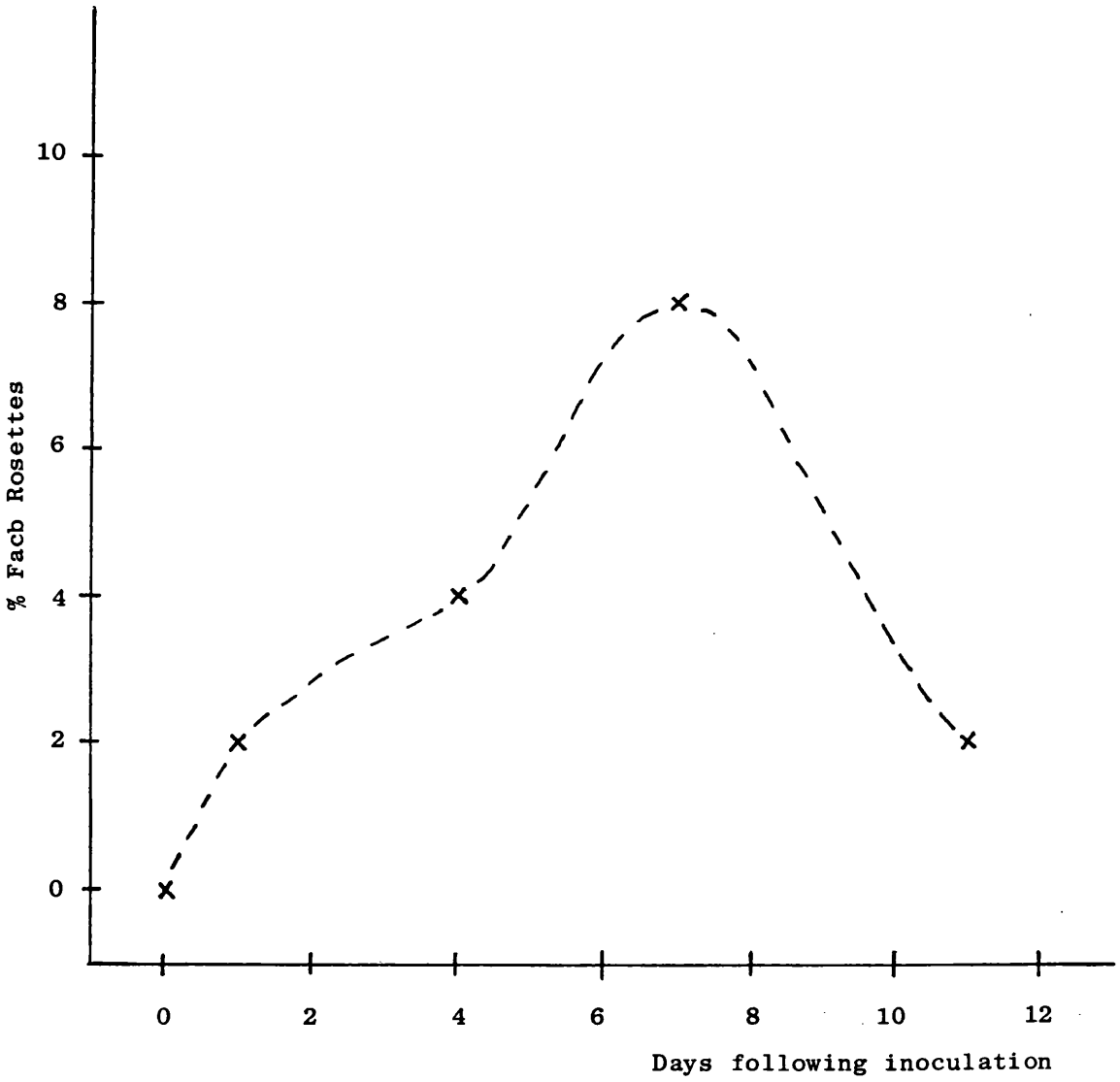
Figure 3.15

PERIPHERAL BLOOD Facb ROSETTE FORMING CELLS

EFFECT OF VACCINATION

Percentage peripheral blood Facb rosette forming cells were enumerated prior to and on several days following inoculation of one healthy subject with an influenza vaccine.

DAYS FOLLOWING VACCINATION	% Facb ROSETTES
0 (before vaccination)	0
1	2
4	4
7	8
11	2


10
8
6
4
2
0
% Facb Rosettes
0
2
4
6
8
10
12
Days following inoculation

CHAPTER FOUR

Fc_{γ} RECEPTORS DETECTED BY FacB ROSETTES: BINDING SPECIFICITIES AND SENSITIVITY TO ENZYME TREATMENT

SECTION I

INTRODUCTION

It was reported in Chapter 3 that patients with RA had increased percentage numbers of $Fc_{\gamma}R$ positive peripheral blood cells, detected by Facb rosettes (Fig. 3.5). It was also shown that a correlation exists between Facb and high avidity EA rosettes (Fig. 3.4). Receptors defined by Facb rosette formation will be termed Facb receptors. Facb receptor bearing cells were also demonstrated in rheumatoid synvoial fluids and those results indicated that there may be some differences in immune complex binding by Facb receptors although that was highly speculative. In this chapter, binding experiments will be reported; these were carried out in order to determine if high avidity EA and Facb rosettes detected the same receptor.

Preliminary experiments were performed to confirm the relationship between EA and Facb rosettes. Depletion of total EA rosettes (Chapter 2; Section II (iii)) resulted in a significant loss of percentage Facb rosettes ($p < 0.05$). Similarly, Facb rosette depletion produced a significant reduction in percentage high avidity EA rosettes ($p < 0.02$); percentage total EA rosettes were lowered but the reduction was not significant (Fig. 4.1). It was noted that Facb rosettes were difficult to deplete due to their fragility; if not handled carefully, particularly during resuspension prior to the second Ficoll-Paque separation, the rosettes tended to break up resulting in an insignificant depletion of rosettes. Depletions were therefore always checked by re-rosetting the interface cells.

Fig. 4.1

ROSETTE DEPLETION EXPERIMENTS

Fig. 4.1 (a)

Mononuclear cell populations (n = 3) were depleted of total EA rosette forming cells (Chapter 2; Section II (iii)). This produced a reduction of Facb rosette forming cells. The results are expressed as percentage rosettes $\pm$ 1 S.D.

	TOTAL EA ROSETTES (%)	Facb ROSETTES (%)
Pre Depletion	16 ± 1	6 ± 1
Post Depletion	3 ± 2	3 ± 1
Significance (Student's t test)	$p < 0.001$	$p < 0.05$

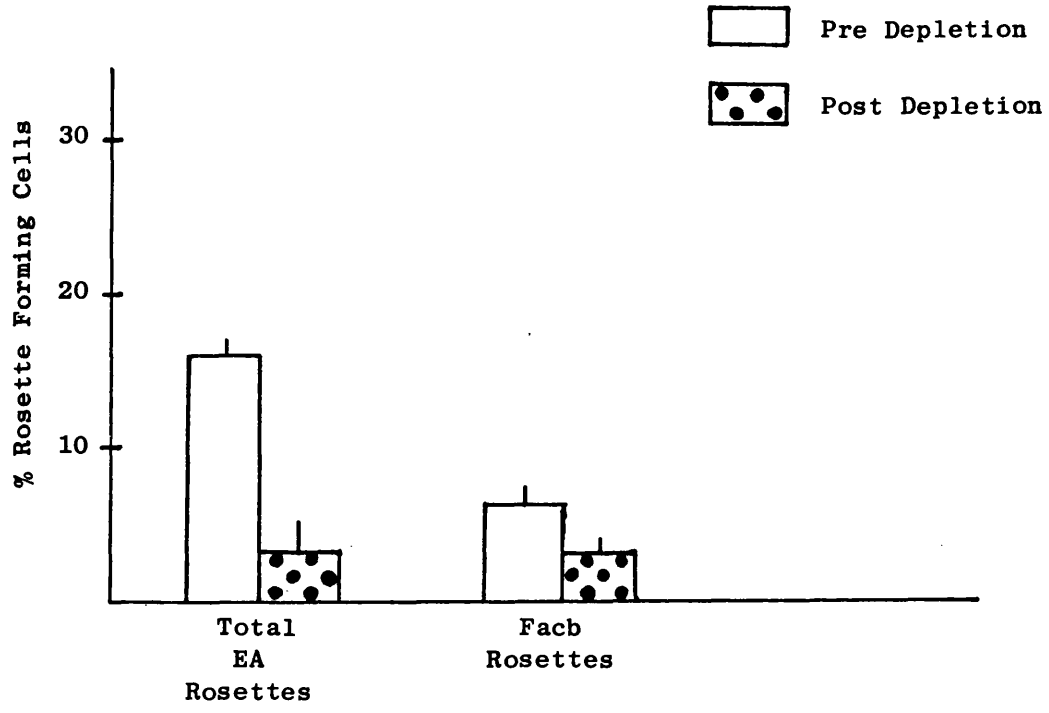
Fig. 4.1 (b)

Mononuclear cell preparations were depleted of Facb rosette forming cells. Percentage total EA rosettes were reduced but the difference was not significant and percentage high avidity EA rosettes were significantly depleted.

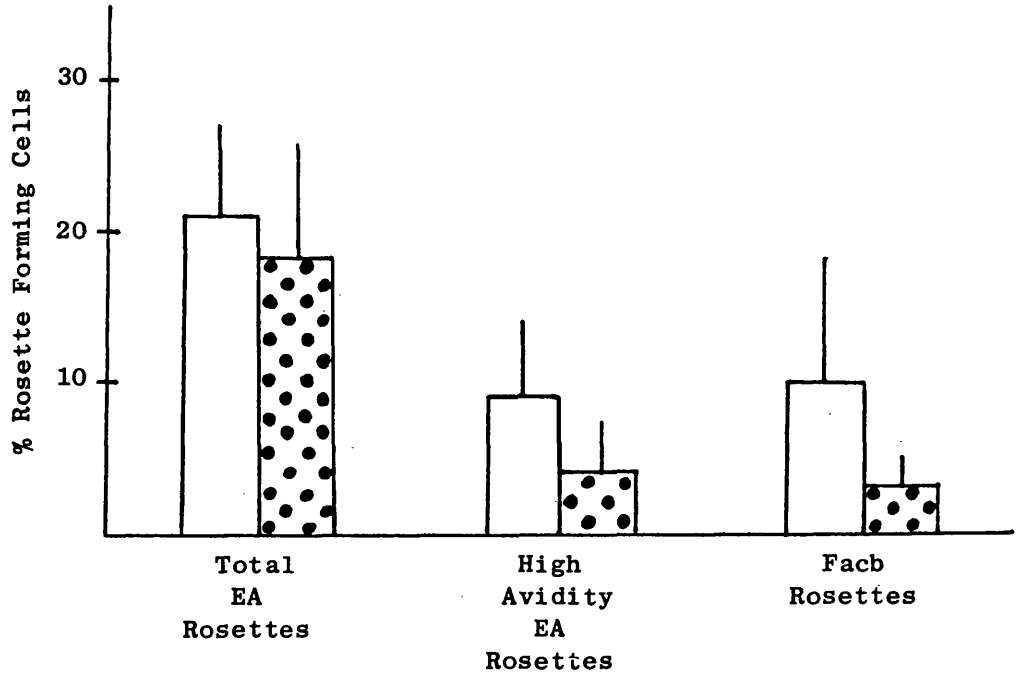
	% ROSETTES $\pm$ 1 S.D.		
	TOTAL EA	HIGH AVIDITY EA	Facb
Pre Depletion	21 ± 6	9 ± 5	10 ± 8
Post Depletion	18 ± 8	4 ± 3	3 ± 2
Number tested	9	7	10
Significance (Student's t test)	NS	$p < 0.02$	$p < 0.05$

NS = not significant

(a)


Pre Depletion
Post Depletion
% Rosette Forming Cells
30
20
10
Total EA Rosettes
Facb Rosettes

(b)


% Rosette Forming Cells
30
20
10
Total EA Rosettes
High Avidity EA Rosettes
Facb Rosettes

SECTION II

Binding Specificity of Fc_γ Receptors Defined by Facb Rosettes and High Avidity EA Rosettes

Human and rabbit native IgG and rabbit IgG fragments were prepared as described previously (Chapter 2; Section II (i)) and stored in a liquid nitrogen refrigerator until required. Myeloma sera of known subclass were used for the preparation of human IgG. These were obtained either from the Royal United Hospital, Bath or as generous gifts from Dr. C. J. Elson (University of Bristol), Dr. P. M. Johnson (University of Liverpool) and Dr. P. I. Gaarder (Statensinstitutt fur Folkehelse, Oslo). The IgG preparations were stored in a liquid nitrogen refrigerator until required. Heat aggregation was performed immediately before use ($63^{o}c$; 10 mins).

Rosette inhibition studies were performed by the addition of competitor protein as follows. A solution of the test protein (10μl or 20μl) or an equal volume of CMFSS was added to the LP3 tube prior to addition of the lymphocytes; the tubes were well mixed and the sensitised erythrocytes added. Rosettes were enumerated as previously described (Chapter 2; Section II (iii)). In early experiments using rabbit IgG, a range of protein concentrations was tested (10-100μg/ml). The optimal final concentration was found to be 40μg/ml and subsequently this concentration was used (10μl of CMFSS or inhibitor protein at 1.6 mg/ml). In each experiment, the percentage rosette formation in the absence of competitor was given the value 100 and the effect of competitor related to this. The mean percentage values of Facb and high avidity EA rosettes

were similar, 11% and 10% respectively. For these experiments, only rheumatoid patients were used since they possessed higher percentage rosette numbers. Accurate measurement of differential binding is difficult using healthy cells due to the low percentage numbers of Facb receptor positive cells; only complete removal of Facb rosette forming cells would probably appear significant (e.g. 4% to $<1\%$). Therefore, the results in this section relate to rheumatoid cells only. For comparative purposes, at least one healthy subject was included in each experiment. All rheumatoid patients were receiving only analgesic or non-steroidal anti-inflammatory therapy.

The results of the competition experiments are shown in Fig. 4.2. The effect of human IgG samples is shown in Fig. 4.2 (a). Both high avidity EA and Facb rosette forming cells showed similar binding specificity with respect to human IgG. Monomeric IgG, isolated from either healthy or rheumatoid sera, did not block either Facb or high avidity EA rosettes. The same preparations when heat aggregated, were strongly inhibitory. Receptors detected by both assays showed strong subclass specificity for IgG1 and IgG3; IgG4 was weakly inhibitory and IgG2 showed no inhibition at the concentration used. At least two different myeloma sera of each IgG subclass were used for these experiments.

Competition experiments carried out using native rabbit IgG and its enzymic digestion products show some differences in binding by Facb receptors and high avidity EA receptors. Native rabbit monomeric IgG and Facb inhibited both rosette assays while Fab fragments showed no inhibition of either assay. However, $F(ab')_2$

Fig. 4.2

COMPETITION EXPERIMENTS WITH HUMAN AND RABBIT IgG AND RABBIT IgG FRAGMENTS

Fig. 4.2 (a)

Inhibition of Facb and high avidity EA rosettes by human IgG. The columns and bars represent the relative mean $\pm$ 1 standard deviation based on a control assay without competitor. The actual percentage Facb and high avidity EA rosette numbers were 11% and 10% respectively. There were no significant differences in binding by the two assay systems.

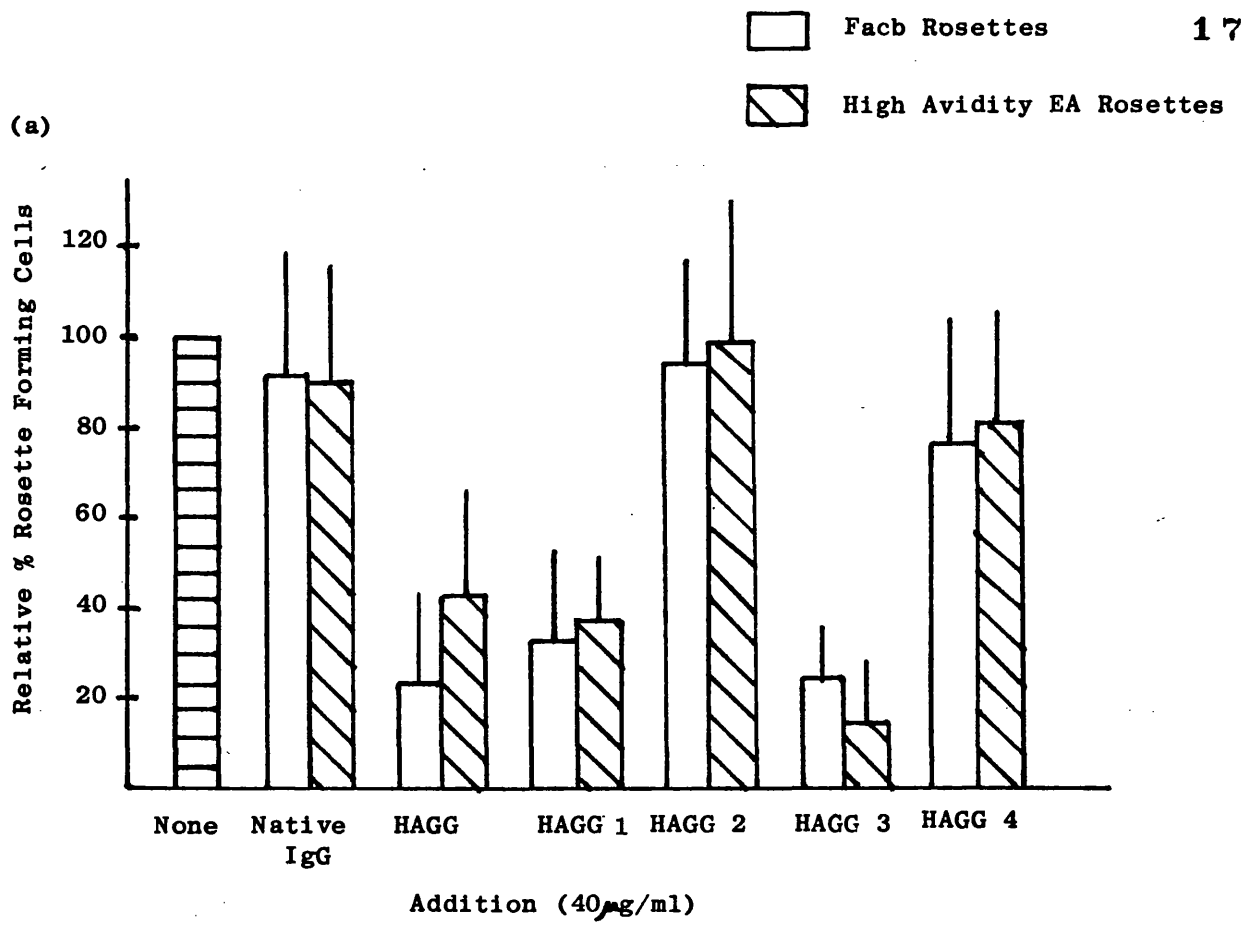
Fig. 4.2 (b)

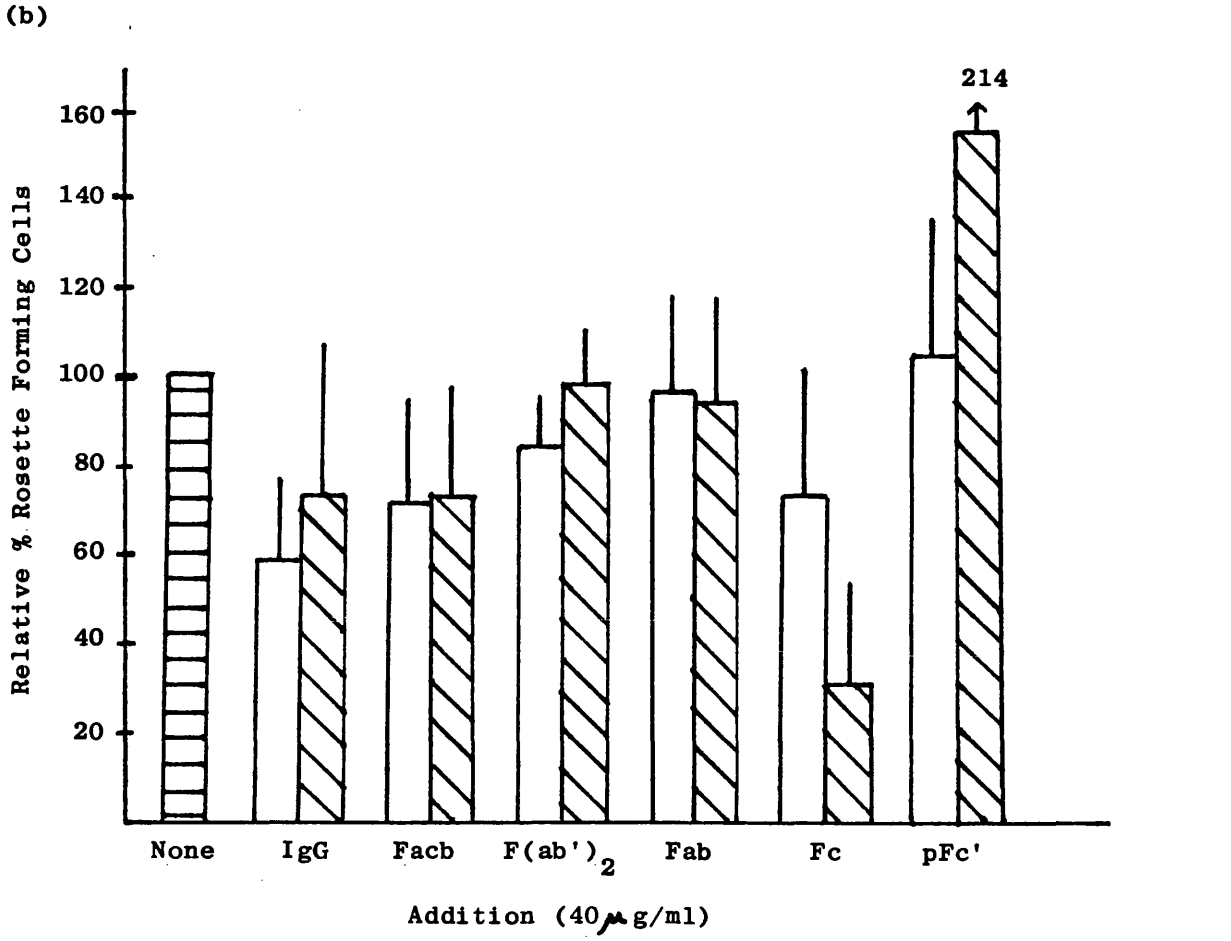
Inhibition of Facb and high avidity EA rosettes by rabbit IgG and IgG fragments. The columns and bars represent the relative mean $\pm$ 1 standard deviation based on a control assay without competitor. The actual percentage Facb and high avidity EA rosette numbers were 11% and 10% respectively.

Significant Results (Student's t test)

Inhibition of Facb rosettes in comparison with high avidity EA rosettes

(i)	$F(ab')_2$	$p < 0.01$	Facb rosettes only
(ii)	Fc	$p < 0.01$	Both assays but high avidity EA more strongly


Facb Rosettes
High Avidity EA Rosettes
(a)
Relative % Rosette Forming Cells
120
100
80
60
40
20
None
Native IgG
HAGG
HAGG 1
HAGG 2
HAGG 3
HAGG 4
Addition (40μg/ml)


(b)
214
Relative % Rosette Forming Cells
160
140
120
100
80
60
40
20
None
IgG
Facb
F(ab')2
Fab
Fc
pFc'
Addition (40μg/ml)

fragments of rabbit IgG were only inhibitory in the Facb rosette assay and this difference in inhibition was significant ($p < 0.01$). In contrast the pFc' fraction, comprising only CH3 domains of IgG, had no effect on Facb rosettes but caused some enhancement of high avidity EA rosette formation. Predictably, the Fc fragment inhibited both rosette assays but the effect was more marked on high avidity EA rosettes ($p < 0.01$). These results indicate that Facb receptor binding may involve the hinge region of IgG. However there is no conclusive proof that Facb and high avidity EA receptors are independent entities.

Carbohydrate binding by $Fc_{\gamma}R$'s was also investigated. It is known, from investigations using myeloma proteins, that immunoglobulin molecules contain oligosaccharide units (Dawson and Clamp, 1968) and such oligosaccharide units have been located in the C-terminal region (Fc fragment) of IgG heavy chains (Franklin, 1960). Should Facb receptors be specifically inhibited by a certain sugar molecule, then it is possible that Facb rosette forming cells could be purified by affinity chromatography. Such procedures have been used for lectin receptors (Hellstrøm et al, 1976). Competition experiments were performed as described for the IgG fragments, using a variety of sugars at a final concentration of 0.1M. Since glucose is a constituent of CMFSS, mononuclear cells were prepared using PBS. It was noted that cell yields were much lower when PBS was used. Thus, the monosaccharide D-glucose and the disaccharide, sucrose were tested as well as two deoxy hexoses and two acetyl hexosamines. The absolute and relative percentage rosettes (calculated as described for IgG fragments) are shown in figure 4.3. In all cases, except that of N-acetyl-D-galactosamine, addition of

Fig. 4.3

COMPETITION EXPERIMENTS : INHIBITION OF Facb AND HIGH AVIDITY EA ROSETTES BY SUGARS

Rheumatoid peripheral blood mononuclear cell preparations (Chapter 2; Section II (ii)) were used for rosette formation incorporating 0.1M concentrations of six saccharide compounds. The columns and bars represent the mean $\pm$ 1 standard deviation. Fig. 4.3 (a) shows the relative Fig. 4.3 (b) the actual percentage rosettes.

Legend

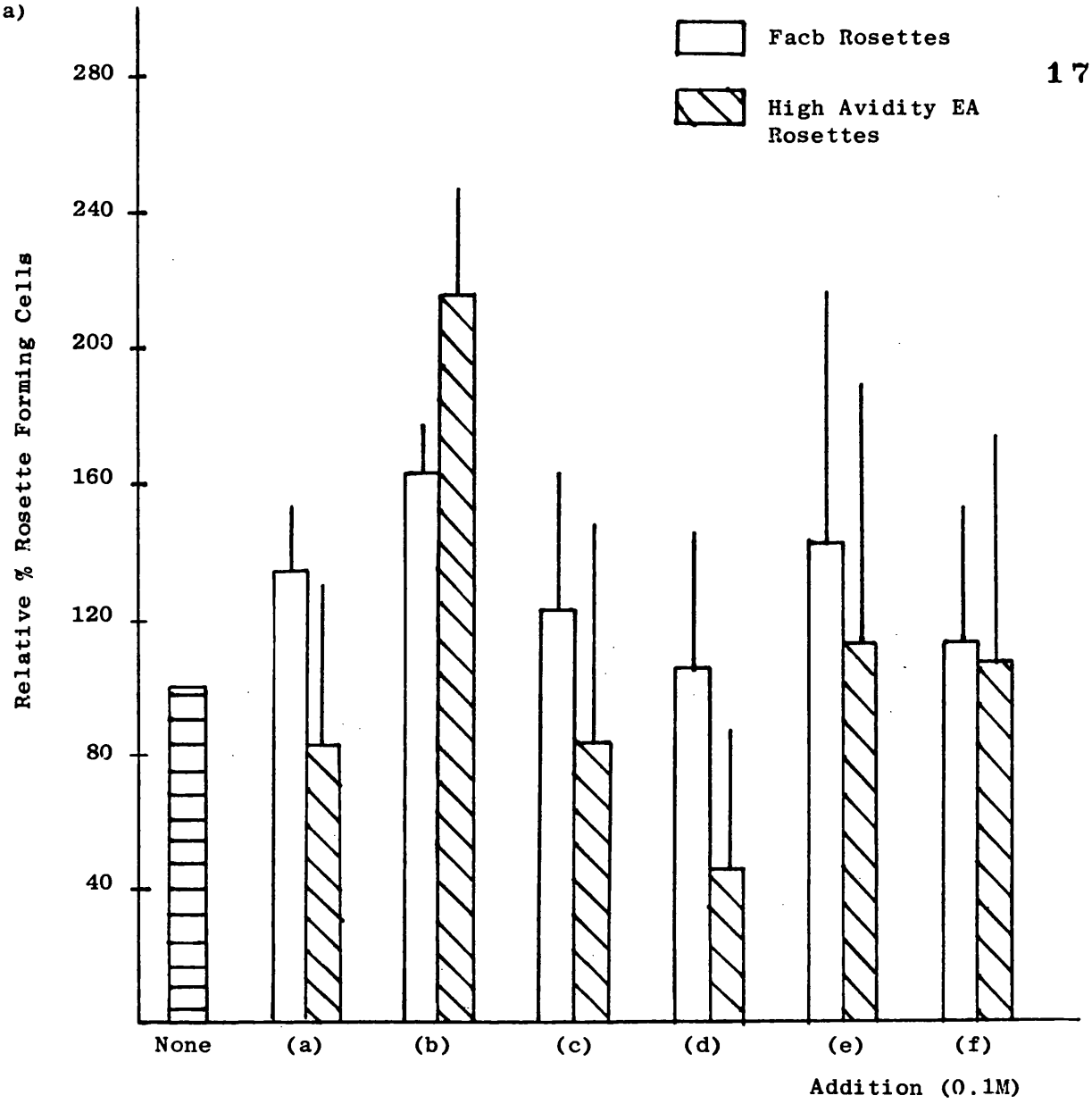
(a) N-acetyl-D-glucosamine
(b) N-acetyl-D-galactosamine
(c) D-glucose
(d) sucrose
(e) 6-deoxy-L-galactose (L-fucose)
(f) 2-deoxy-D-glucose

Results

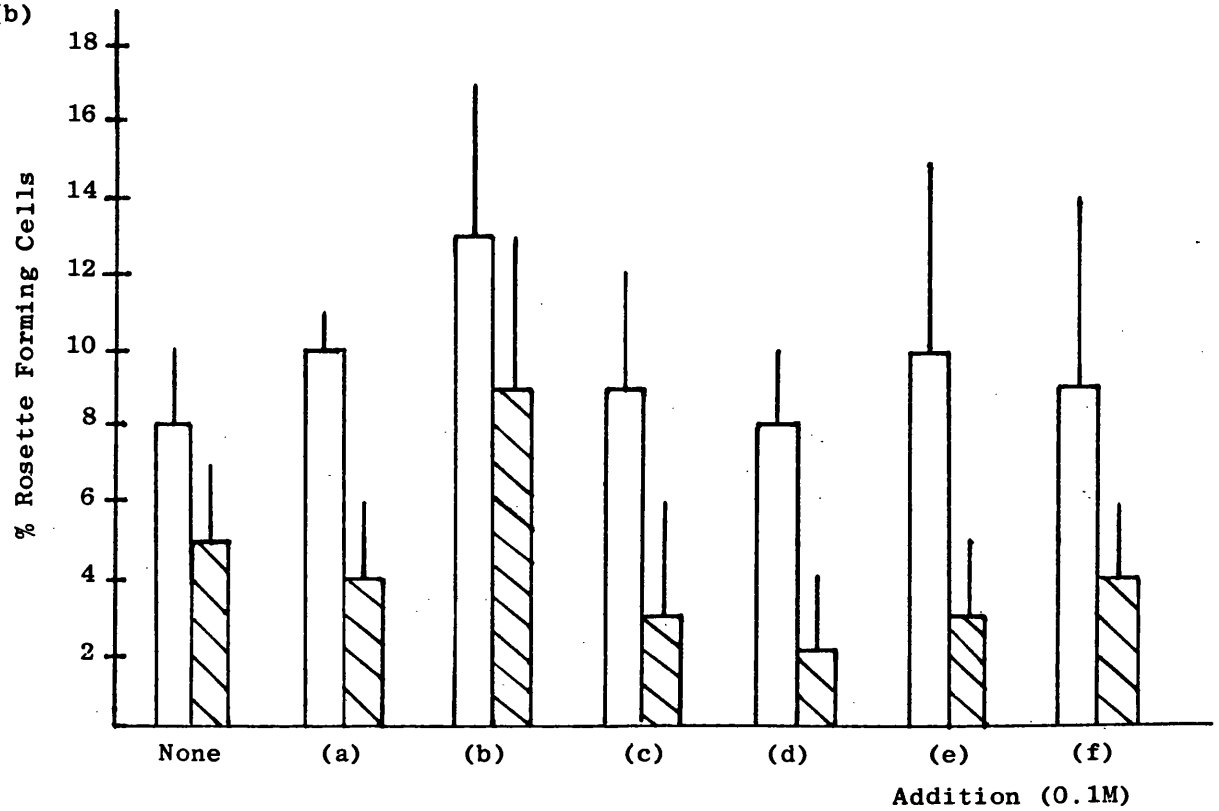
		% ROSETTES $\pm$ 1 S.D.		% RELATIVE ROSETTES $\pm$ 1 S.D.	
ADDITION (0.1M)	NO. TESTED	HIGH AVIDITY EA	Facb	HIGH AVIDITY EA	Facb
None	3	5 $\pm$ 2	8 $\pm$ 2	100	100
(a)	3	4 $\pm$ 2	10 $\pm$ 1	83 $\pm$ 47	133 $\pm$ 19
(b)	3	9 $\pm$ 4	13 $\pm$ 4	217 $\pm$ 31	163 $\pm$ 15
(c)	3	3 $\pm$ 3	9 $\pm$ 3	83 $\pm$ 66	123 $\pm$ 40
(d)	3	2 $\pm$ 2	8 $\pm$ 2	46 $\pm$ 41	106 $\pm$ 40
(e)	3	3 $\pm$ 2	10 $\pm$ 5	113 $\pm$ 77	143 $\pm$ 73
(f)	3	4 $\pm$ 2	9 $\pm$ 5	108 $\pm$ 66	113 $\pm$ 40

There were no significant differences (Student's t test) between high avidity EA and Facb rosettes with respect to inhibition.

(a)


Facb Rosettes
High Avidity EA Rosettes
Relative % Rosette Forming Cells
280
240
200
160
120
80
40
None
(a)
(b)
(c)
(d)
(e)
(f)
Addition (0.1M)

(b)


% Rosette Forming Cells
18
16
14
12
10
8
6
4
2
None
(a)
(b)
(c)
(d)
(e)
(f)
Addition (0.1M)

sugar molecules to 0.1M tended to decrease high avidity EA rosettes and increase Facb rosettes. The acetyl hexosamine, N-acetyl-D-galactosamine enhanced both Facb and high avidity EA rosettes but this increase was not significant. The results are based on only three experiments but they suggest that D-glucose has little effect on rosette formation at 0.1M; the salt solution, CMFSS, contains only 1.6 g/litre of glucose (< 10mM). None of the sugars tested gave complete inhibition of rosette formation.

S E C T I O N I I I

Fc Receptors Detected by Facb and High Avidity EA Rosettes : Effect of Enzyme Treatment

Fc receptors have been characterised by their susceptibility to proteolysis (Chapter 1; Section IV (ii)). Rheumatoid and healthy mononuclear cell preparations were treated with neuraminidase, trypsin or phospholipase C. The experimental details are given in Chapter 2, Section II (iv). EA and Facb rosettes were prepared before and after enzyme treatment in all cases. E rosettes were set up prior to and following neuraminidase and trypsin treatment. Only one concentration of each of these enzymes was used. In the case of phospholipase C, three different enzyme concentrations were assayed but E rosettes were not set up.

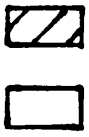
The effect of proteolysis on rosette formation using trypsin is shown in figure 4.4. Incubation of rheumatoid cells at 37^{o}c produced a drop in all rosette forming cells measured but the differences were not significant. Healthy cells, however, showed a significant

Fig. 4.4

EFFECT OF TRYPSIN ON MONONUCLEAR CELLS

E, EA and Facb rosettes were prepared using healthy and rheumatoid mononuclear cells before and after incubation at 37°C and following proteolysis with trypsin (Chapter 2; Section II (iv)). The columns and bars represent the mean percentage rosette number ± 1 standard deviation. Results are given below.

Legend



RA

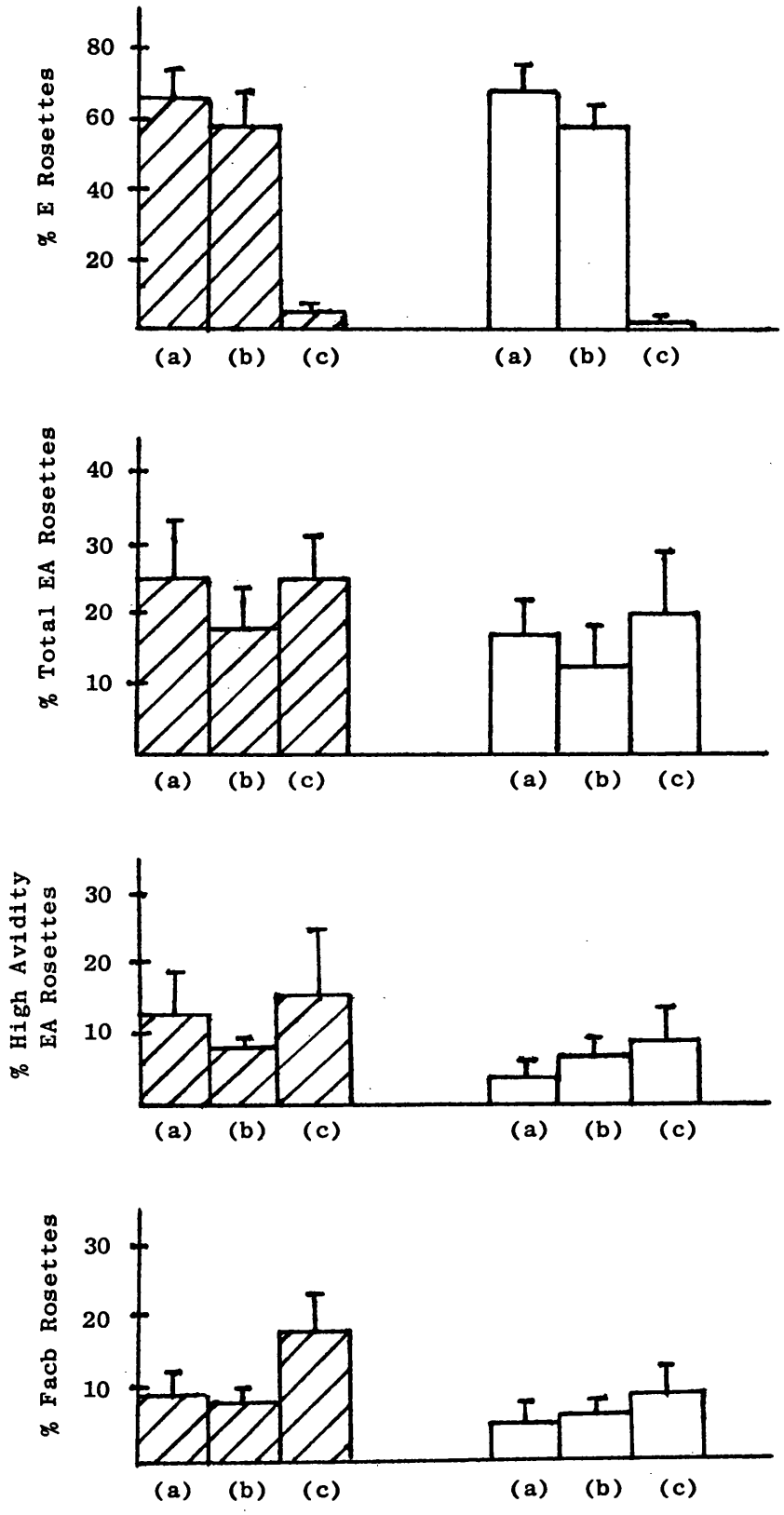
Healthy

(a) = before 37°C incubation
(b) = after 37°C incubation
(c) = following trypsin treatment

Results

		% ROSETTES ± 1 S.D.				
		E	TOTAL EA	HIGH AVIDITY EA	Facb	YIELD (%)
RA	(a)	66 ± 9	25 ± 8	13 ± 6	9 ± 3	-
	(b)	58 ± 10	18 ± 6	8 ± 1 *	8 ± 2	64 ± 20
	(c)	4 ± 1 **	25 ± 6	16 ± 9 *	18 ± 5	61 ± 9
		(n=6)	(n=6)	(n=5)	(n=6)	
Healthy	(a)	68 ± 7 X	17 ± 5	4 ± 2 *	5 ± 3	-
	(b)	57 ± 7 X	13 ± 5	7 ± 2 *	6 ± 2	72 ± 9
	(c)	2 ± 1 **	20 ± 5	9 ± 5	9 ± 4	65 ± 19
		(n=5)	(n=5)	(n=5)	(n=5)	

Significance (Student's t test) X $p < 0.05$
* $p < 0.01$
** $p < 0.001$


% E Rosettes
80
60
40
20
(a) (b) (c) (a) (b) (c)
% Total EA Rosettes
40
30
20
10
(a) (b) (c) (a) (b) (c)
% High Avidity EA Rosettes
30
20
10
(a) (b) (c) (a) (b) (c)
% Facb Rosettes
30
20
10
(a) (b) (c) (a) (b) (c)

loss of E rosettes ($p < 0.05$) and a parallel significant increase in high avidity EA rosettes ($p < 0.05$); these results may reflect cell death since only live T cells form E rosettes (WHO report, 1974). Trypsin virtually eliminated E rosette formation in both rheumatoid and healthy populations. Rheumatoid Facb rosettes were significantly increased ($p < 0.01$); although high avidity EA rosettes were also increased, the difference was not significant. Healthy trypsinised cells showed no significant percentage differences in Facb and high avidity EA rosettes. It is possible that the increase observed with rheumatoid cells merely reflects removal of immune complexes from blocked receptors. Thus E rosette forming cells have trypsin sensitive sheep erythrocyte receptors while the Fc γ receptor positive cells are trypsin resistant.

Neuraminidase cleaves sialic acid residues from glycosylated molecules. The effect of neuraminidase on rosette formation is shown in figure 4.5. Results from both healthy and rheumatoid subjects are combined since only 2 rheumatoid and 2-3 control subjects were used. The results appeared to be very similar. Predictably, neuraminidase enhanced E rosettes; this agrees with the findings of Hammarstrøm et al (1973). However, the enhancement was not significant. All FcγR positive cells measured by the Facb and EA rosette assays were significantly increased. Thus FcγR's on peripheral blood cells are neuraminidase resistant in that neuraminidase does not abolish EA or Facb rosette formation.

The effect of phospholipase C on peripheral blood FcγR bearing lymphocytes is shown in figure 4.6. Cell viabilities dropped dramatically with increasing concentrations of phospholipase C. The

Fig. 4.5

NEURAMINIDASE TREATMENT OF PERIPHERAL BLOOD MONONUCLEAR CELLS : EFFECT ON ROSETTE FORMATION

Mononuclear cells prepared on Ficoll Paque (Chapter 2; Section II (ii)) were treated with neuraminidase (Chapter 2; Section II (iv). Rosettes were set up before and after treatment. The columns and bars represent the mean $\pm$ 1 standard deviation.

	% ROSETTES $\pm$ 1 S.D.			
	E	TOTAL EA	HIGH AVIDITY EA	Facb
Untreated	64 $\pm$ 12	20 $\pm$ 8	12 $\pm$ 4	13 $\pm$ 4
Neuraminidase Treated	75 $\pm$ 13	36 $\pm$ 7	18 $\pm$ 2	25 $\pm$ 4
No. Tested	5	5	4	5
Significance (Student's t test)	NS	$p < 0.01$	$p < 0.05$	$p < 0.01$

NS = not significant

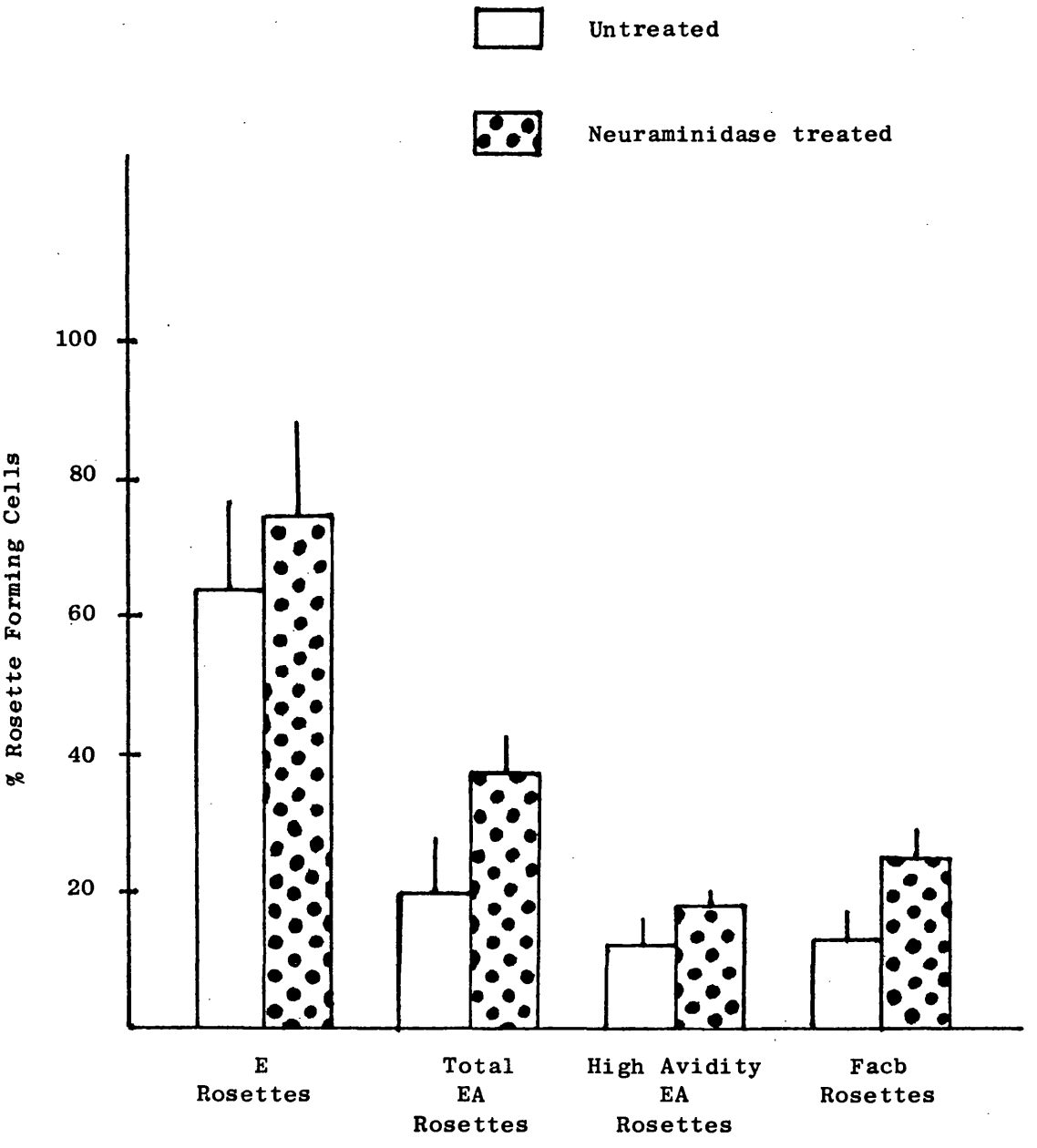

Untreated
Neuraminidase treated
% Rosette Forming Cells
100
80
60
40
20
E Rosettes
Total EA Rosettes
High Avidity EA Rosettes
Facb Rosettes

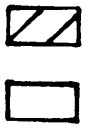
Fig. 4.6

PHOSPHOLIPASE C TREATMENT OF HEALTHY AND RHEUMATOID PERIPHERAL BLOOD MONONUCLEAR CELLS

EFFECT ON $Fc_\gamma R$ POSITIVE CELLS

Mononuclear cells prepared by isopycnic centrifugation (Chapter 2; section II (ii)) were treated with phospholipase C (Chapter 2; Section II (iv)). Percentage $Fc_\gamma R$ positive cells were measured before and after treatment. The results are shown opposite. The columns and bars represent the mean $\pm$ 1 standard deviation.

Legend



RA

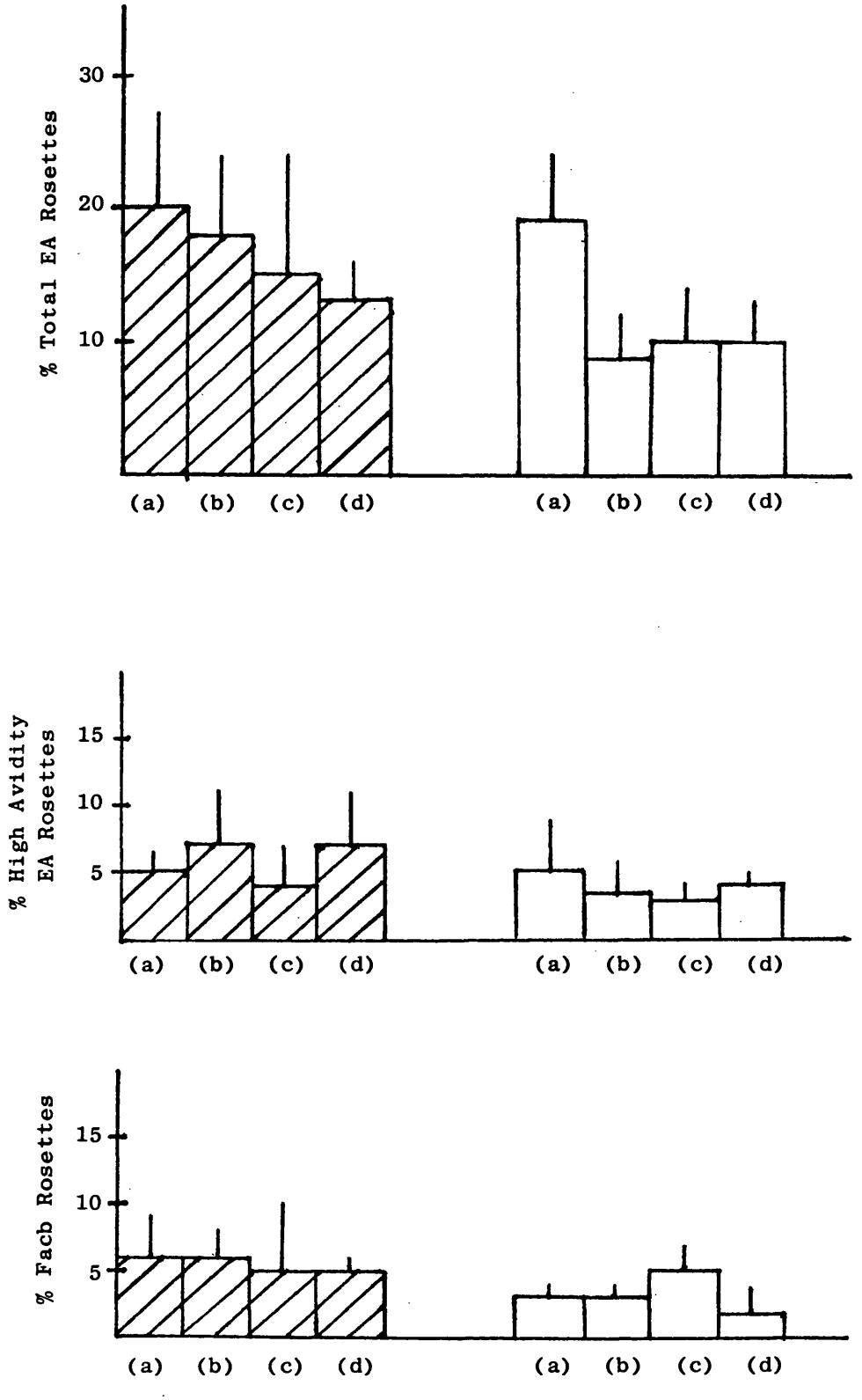
Healthy

(a) no enzyme
(b) 0.1 U/ml phospholipase C
(c) 0.5 U/ml phospholipase C
(d) 1.0 U/ml phospholipase C

Results

		% ROSETTES $\pm$ 1 S.D.				
		VIABILITY %	TOTAL EA	HIGH AVIDITY EA	Facb	NO. TESTED
RA	(a)	96 $\pm$ 3	20 $\pm$ 7	5 $\pm$ 3	6 $\pm$ 3	10
	(b)	88 $\pm$ 15	18 $\pm$ 6	7 $\pm$ 4	6 $\pm$ 2	10
	(c)	75 $\pm$ 11	15 $\pm$ 9	4 $\pm$ 3	5 $\pm$ 5	6
	(d)	60 $\pm$ 10	13 $\pm$ 3	7 $\pm$ 4	5 $\pm$ 1	3
Healthy	(a)	95 $\pm$ 1	19 $\pm$ 5	5 $\pm$ 4	3 $\pm$ 1	6
	(b)	80 $\pm$ 8	9 $\pm$ 3	3 $\pm$ 3	3 $\pm$ 1	6
	(c)	67 $\pm$ 23	10 $\pm$ 4	3 $\pm$ 1	5 $\pm$ 2	4
	(d)	40 $\pm$ 11	10 $\pm$ 3	4 $\pm$ 1	2 $\pm$ 2	2

Significant Results (Student's t test) Healthy (a) Vs (b) $p < 0.01$
(a) Vs (c) $p < 0.02$


% Total EA Rosettes
30
20
10
(a) (b) (c) (d)
(a) (b) (c) (d)
% High Avidity EA Rosettes
15
10
5
(a) (b) (c) (d)
(a) (b) (c) (d)
% Facb Rosettes
15
10
5
(a) (b) (c) (d)
(a) (b) (c) (d)

results indicate that both rheumatoid and healthy Facb and high avidity EA rosette forming cells have phospholipase C resistant $Fc_{\gamma}R$'s. The effect of phospholipase C on percentage total EA rosettes was different in rheumatoid and healthy subjects. Percentage rheumatoid EA rosette forming cells fell progressively with increasing concentrations of enzyme while percentage healthy total EA rosettes were sensitive to low concentrations of enzyme (0.1 U/ml). Higher concentrations of phospholipase C produced no further loss of healthy EA rosette forming cells. Significance levels measured by the Student's t test are given in figure 4.6. The loss of significance at 1.0 Unit/ml using healthy cells probably reflects the low numbers (n=2).

CHAPTER FIVE

CHARACTERISATION OF THE FACB RECEPTOR POSITIVE CELL

SECTION I

INTRODUCTION

In Chapters 3 and 4, it was shown that circulating peripheral mononuclear cells bear $Fc_{\gamma}R$'s which recognise the CH2 region of IgG and these cells are present in high percentages in rheumatoid patients. The receptor appears to have a high avidity for IgG and binding is specific for the CH2 region, although some involvement of the hinge region may occur. This receptor is not exclusive to rheumatoid patients since raised percentages are occasionally detected in normal subjects and also occur after skin testing of primed individuals.

In this chapter, attempts to characterise the Facb receptor positive cell will be accounted.

SECTION II

PHAGOCYTE DEPLETION OF MONONUCLEAR CELL PREPARATIONS : EFFECT ON Facb ROSETTE FORMATION

Ficoll-Paque separation of whole blood results in lymphocyte populations contaminated with monocytes since the density of monocytes falls within the range of lymphocyte densities; polymorphonuclear leucocytes may also be present in smaller numbers. It is possible that the raised percentage numbers of Facb rosetting cells detected in rheumatoid peripheral blood are due to raised numbers of contaminating monocytes or granulocytes since both cell types bear $Fc_{\gamma}R$'s.

Phagocytes were removed from mononuclear cell preparations either by adherence to plastic tissue culture flasks (Nunc; Gibco Bio Cult Ltd.) or by treatment with carbonyl iron. Cell smears stained with α-naphthyl-acetyl-(non-specific) esterase show that incubation for 30 minutes at 37^{o}C or addition of 20 mg/ml of carbonyl iron removed monocytes (Tables 5.1 and 5.2). Table 5.3 demonstrates that removal of plastic adherent or phagocytic cells had no significant effect on Facb rosette formation. However, cells obtained after plastic adherence showed much disruption and therefore carbonyl iron appears to be a better method of phagocyte removal. Using either method, lymphocyte yeilds were low (< 50%). A purified monocyte population (prepared on Percoll by Miss C. Minty, RNHRD) consisting of 88% monocytes was used for Facb rosette formation; only 12% of the cells formed Facb rosettes (Table 5.4). Thus, a contaminating monocyte population of 20-30% would produce only 2-3% Facb rosettes. Similarly, using purified polymorphonuclear cell preparations (prepared on Percoll by Miss C. Minty, RNHRD), Facb rosettes were detected in a mean of only 10% of the cells (Table 5.5). One synovial fluid polymorphonuclear cell preparation, harvested from the base of a Ficoll-Paque gradient after lymphocyte separation, was also measured (Table 5.5); the Facb rosette percentage numbers were very low (3%).

The low percentage numbers of Facb rosettes obtained using purified phagocyte preparations may have been obtained because the sensitising antibody concentrations are not optimal for monocyte and granulocyte rosettes. Therefore, it cannot be assumed that only low numbers of phagocytes form Facb rosettes. However, it can be stated that raised percentages of FacbR positive lymphocytes observed in RA in the current study are not due to the effects of such cells.

Table 5.1

MONOCYTE DEPLETION FROM MONONUCLEAR

CELL SUSPENSION BY ADHERENCE TO PLASTIC

SUBJECT	% MONOCYTES	
	PRE INCUBATION	POST INCUBATION
RA 1	11	1
RA 2	12	1
RA 3	1	1
RA 4	10	1
HEALTHY 1	9	2
HEALTHY 2	7	2

Tabe 5.2

MONOCYTE DEPLETION FROM MONONUCLEAR

CELL SUSPENSION BY CARBONYL IRON

SUBJECT	% MONOCYTES			
	CARBONYL IRON CONC. (mg/ml)			
	0	1	5	20
RA	8	< 1	< 1	< 1
HEALTHY	23	8	< 1	< 1

Table 5.3

EFFECT OF MONOCYTE DEPLETION ON

Facb ROSETTE FORMATION BY MONONUCLEAR

CELL SUSPENSIONS

METHOD OF MONOCYTE DEPLETION	NUMBER TESTED	% Facb ROSETTES ± 1 S.D.	
		PRE DEPLETION	POST DEPLETION
Plastic adherence	16	6 ± 3	5 ± 3
Carbonyl Iron (20 mg/ml)	24	11 ± 6	11 ± 6

Table 5.4

Facb ROSETTE FORMATION BY PURIFIED MONOCYTES

PURIFIED MONOCYTE POPULATION	TOTAL EA ROSETTES	HIGH AVIDITY EA ROSETTES	Facb ROSETTES
88% Monocytes 11% Lymphocytes 1% Neutrophils	60%	4%	12%

Table 5.5

Facb ROSETTE FORMATION BY PURIFIED POLYMYOPHONUCLEAR CELLS

SOURCE	SUBJECT	PURITY	TOTAL EA ROSETTES	HIGH AVIDITY EA ROSETTES	Facb ROSETTES
Peripheral Blood	RA 1	99%	69%	2%	28%
	RA 2	99%	79%	3%	3%
	Healthy 1	99%	60%	11%	12%
	Healthy 2	99%	75%	0%	14%
	Healthy 3	99%	79%	2%	2%
	Healthy 4	99%	68%	6%	3%
			Mean = $72^{+}_{-}7$	Mean = $4^{+}_{-}4$	Mean = $10^{+}_{-}9$
Synovial Fluid	RA	99%	44%	5%	3%

SECTION III

CHARACTERISATION OF THE LYMPHOCYTE SUBPOPULATION FORMING Facb ROSETTES

As described previously, $Fc_{\gamma}R$'s are present on many cell types (Chapter 1, Section IV). Thus subsets of T cells, B cells and null cells all bear $Fc_{\gamma}Rs$ and these receptors can also be detected on certain virus transformed cells and at various stages during cell differentiation. In this section, experiments will be described which attempt to characterise the lymphocyte subpopulation bearing Facb receptors.

(i) T Cells

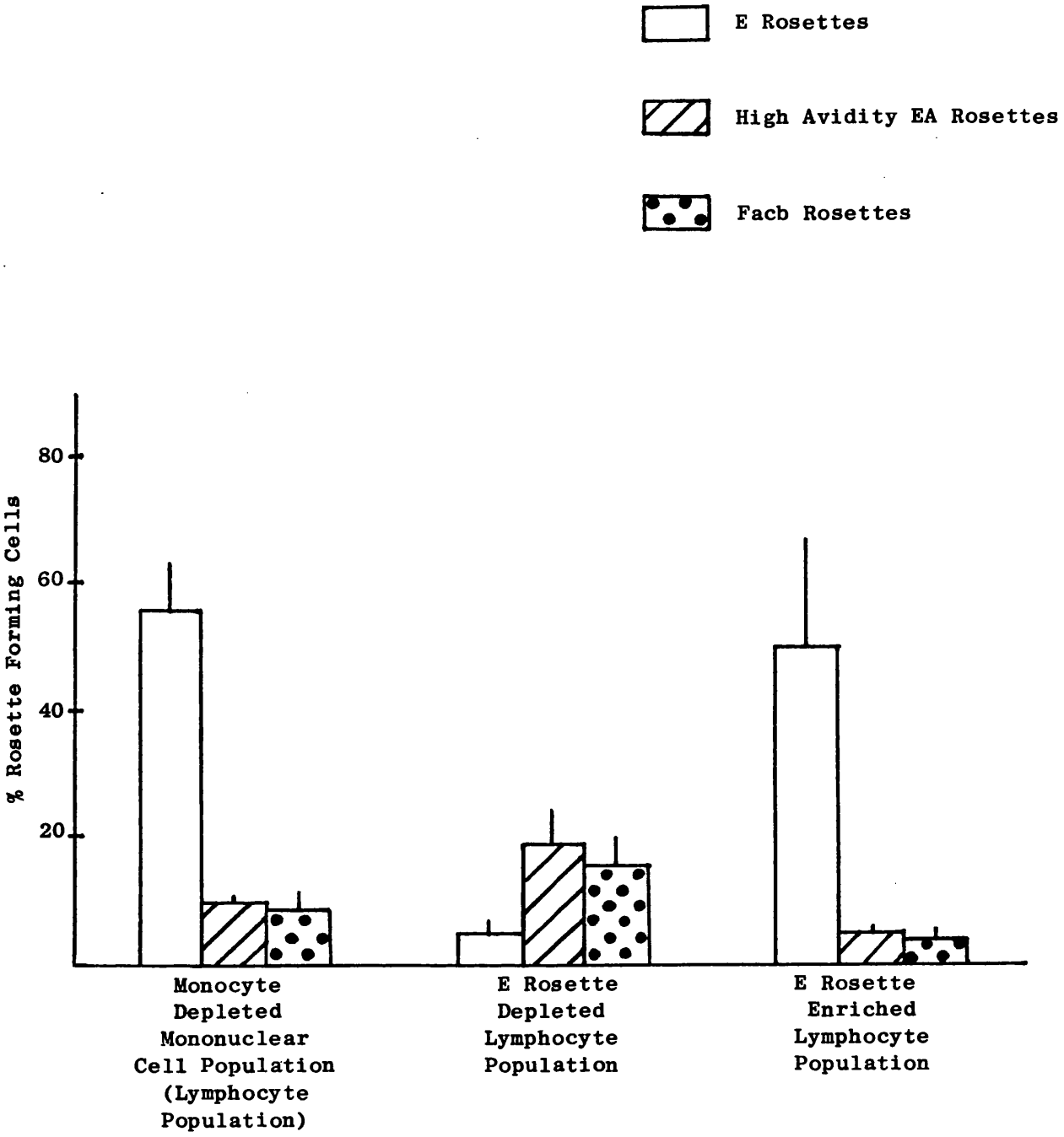
T cells are commonly characterised by their ability to form rosettes with sheep erythrocytes (E rosettes). Mononuclear cells prepared on Ficoll-Paque were depleted of phagocytic cells by treatment with carbonyl iron. This lymphocyte population was then depleted of E rosette forming cells using neuraminidase treated sheep erythrocytes (Chapter 2, Section II (iii)). Both the E rosette enriched and E rosette depleted populations were harvested and used for rosette formation. The results of two such experiments are presented in figure 5.1. E rosette depletion increased the percentage of Facb rosette forming cells while enrichment of E rosette positive cells decreased the percentage Facb receptor positive cells. The results show that the majority, if not all, of the Facb receptor positive cells do not form E rosettes.

Fig. 5.1

E ROSETTE DEPLETION OF MONONUCLEAR CELL PREPARATIONS : EFFECT ON Facb RECEPTOR POSITIVE CELLS

Phagocytic cells were removed from mononuclear cell preparations by plastic adherence. This lymphocyte population was then depleted of E rosette positive cells. E, high avidity EA and Facb rosettes were set up on the lymphocyte population and on both E rosette enriched and depleted cell populations. E rosette depletion produced an enrichment of Facb receptor positive cells but this is not significant.

	% ROSETTES			
	E	HIGH AVIDITY EA	Facb	NO. TESTED
Non Adherent Cells	56 $\pm$ 8	10 $\pm$ 1	9 $\pm$ 2	2
Non E-rosetting Cells	5 $\pm$ 2	19 $\pm$ 6	16 $\pm$ 5	2
E-rosetting Cells	50 $\pm$ 17	5 $\pm$ 1	4 $\pm$ 1	2


E Rosettes
High Avidity EA Rosettes
Facb Rosettes
% Rosette Forming Cells
80
60
40
20
Monocyte Depleted Mononuclear Cell Population (Lymphocyte Population)
E Rosette Depleted Lymphocyte Population
E Rosette Enriched Lymphocyte Population

Helix pomatia lectin Sepharose can be used for the purification of T cells (Hellstrøm et al, 1976). Mononuclear cell preparations were treated with neuraminidase and applied to a column of Helix pomatia lectin Sepharose. The cells were eluted with various buffers as described in Chapter 2, Section II (v). Rosettes were prepared at each stage of the procedure. The results are shown in figure 5.2. A group of four rheumatoid patients and a group of four healthy subjects were used in these experiments. Both groups behaved identically and thus the results are presented as a whole. E rosette positive cells were significantly decreased in the unbound fraction ($p < 0.001$) in comparison with untreated cells; the bound fraction (fraction 5) showed significantly increased numbers of T cells ($p < 0.05$). In comparison, total EA rosettes were significantly enhanced in the unbound fraction ($p < 0.001$) and significantly depleted in the bound fraction ($p < 0.01$). Both Facb and high avidity EA rosettes followed a similar pattern to total EA rosettes. These results also indicate that Facb receptor positive cells are not T cells.

(ii) B and Null Cells

The most popular method of B cell identification is by measurement of surface membrane immunoglobulin (sIg). Fig. 5.3 shows experiments used to correlate Facb receptor positive cells with B cells. Calf erythrocytes were chromic chloride conjugated to normal sheep IgG or to sheep IgG anti-human light chain (prepared by Mr. A. Ahmed, Department of Biochemistry, Bath University). These erythrocytes were used to deplete immunoglobulin bearing cells. Facb rosettes were prepared on untreated and B cell depleted

Fig. 5.2

PURIFICATION OF T CELLS USING HELIX POMATIA LECTIN SEPHAROSE : A SEARCH FOR Facb RECEPTORS ON PERIPHERAL BLOOD T CELLS

The experimental procedure is given in Chapter 2, Section II (v). The combined results of four rheumatoid and four healthy subjects are presented. Unbound cells were enriched in Facb receptor positive cells while the bound fraction contained low percentages of Facb receptor positive cells. The effect of neuraminidase treatment has been reported in Chapter 4.

Legend

(1) Unfractionated mononuclear cell population
(2) Neuraminidase treated mononuclear cells
(3) Unbound cells
(4) Weakly bound cells
(5) Bound cells

RESULTS

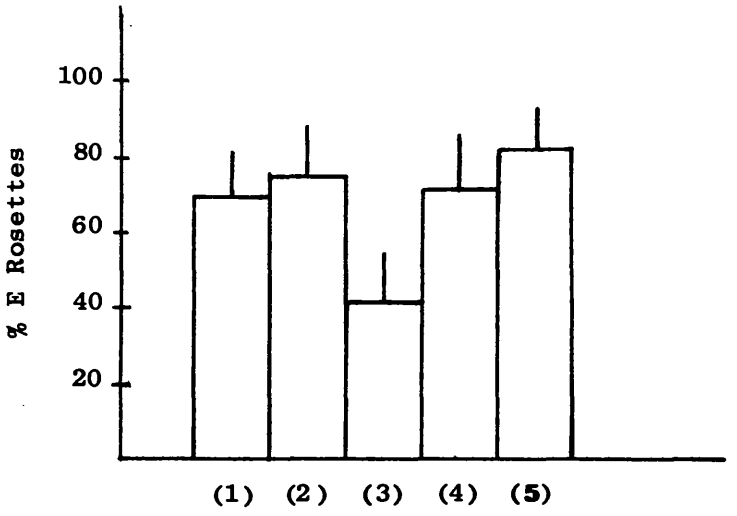
% ROSETTE FORMING CELLS $\pm$ 1 S.D.

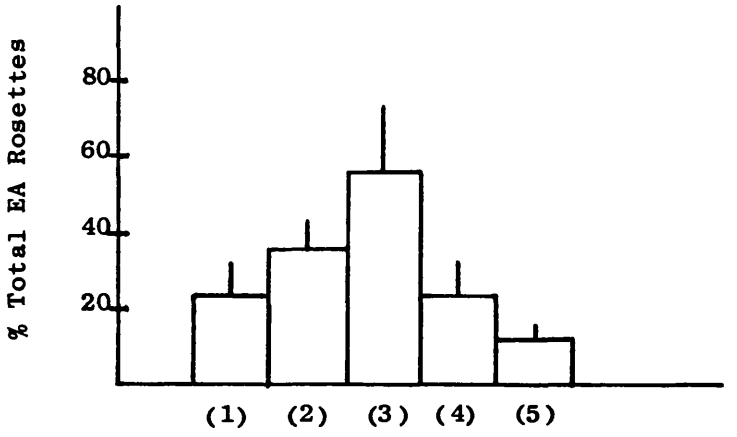
FRACTION	E	TOTAL EA	HIGH AVIDITY EA	Facb
(1)	$69^{+}_{-}12$ (8)	$23^{+}_{-}9$ (8)	$14^{+}_{-}7$ (7)	$15^{+}_{-}5$ (6)
(2)	$75^{+}_{-}13$ (5)	$36^{+}_{-}7$ (5)	$18^{+}_{-}2$ (4)	$25^{+}_{-}4$ (5)
(3)	$42^{+}_{-}13$ (8)	$27^{+}_{-}17$ (8)	$36^{+}_{-}13$ (7)	$44^{+}_{-}6$ (6)
(4)	$72^{+}_{-}14$ (8)	$24^{+}_{-}8$ (9)	$15^{+}_{-}7$ (7)	$17^{+}_{-}9$ (6)
(5)	$82^{+}_{-}11$ (8)	$11^{+}_{-}4$ (8)	$6^{+}_{-}4$ (7)	$6^{+}_{-}4$ (6)

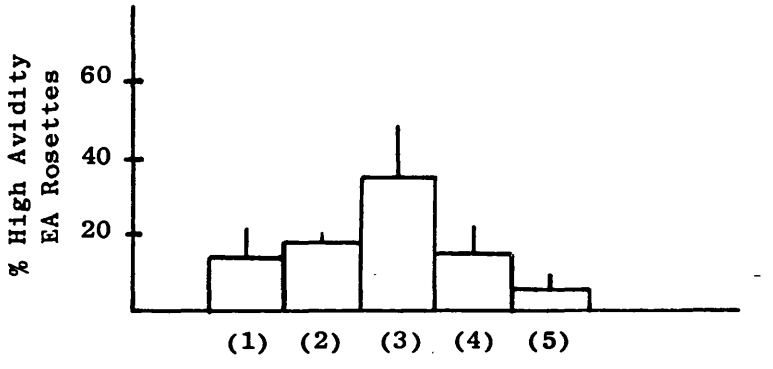
The numbers in brackets after percentage rosettes refer to the number of samples tested.

Significant Results (Student's t test)

	① Vs ③	① Vs ⑤
E Rosettes	$p < 0.001$	$p < 0.05$
Total EA Rosettes	$p < 0.001$	$p < 0.01$
High Avidity EA Rosettes	$p < 0.01$	$p < 0.05$
Facb Rosettes	$p < 0.001$	$p < 0.01$


% E Rosettes
100
80
60
40
20
(1) (2) (3) (4) (5)


% Total EA Rosettes
80
60
40
20
(1) (2) (3) (4) (5)


% High Avidity EA Rosettes
60
40
20
(1) (2) (3) (4) (5)

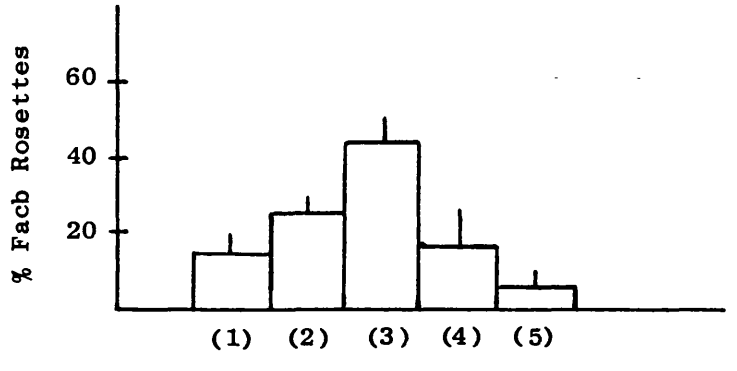

% Facb Rosettes
60
40
20
(1) (2) (3) (4) (5)

Fig. 5.3

A SEARCH FOR Facb RECEPTORS ON RHEUMATOID PERIPHERAL BLOOD B CELLS

Chromic chloride conjugated erythrocytes (Calf RBC) were used for the preparation of cell populations depleted of light chain positive lymphocytes (Chapter 4, Section III (iii)). Light chain and Facb rosettes and sIg were measured before and after depletion. The results are shown opposite; the columns and bars represent the means and standard deviations of the various cell populations.

RESULTS

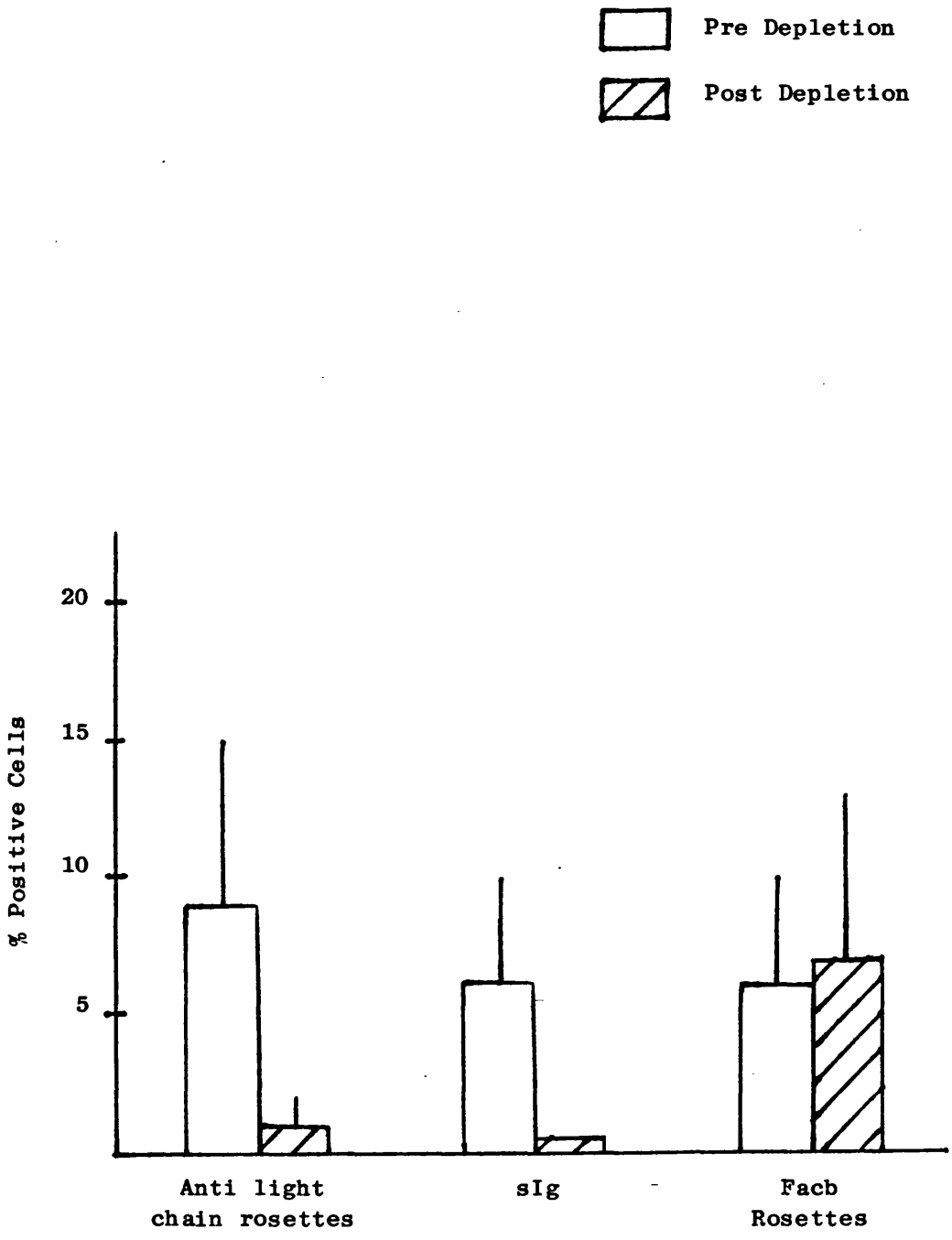
(1) Calf RBC - Sheep IgG (% rosettes)

(2) Calf RBC - Sheep anti human light chain (% rosettes)

sIg % B cells (measured by immunofluorescence; Chapter 2, Section II v)

SUBJECT	Pre Depletion				
	(1)	(2)	(2)-(1)	sIg	Facb
RA 1	2	12	10	4	4
RA 2	2	5	3	12	3
RA 3	3	21	18	2	14
RA 4	2	8	6	4	4
mean $\pm$ 1 S.D.	-	-	$9^{+}_{-}6$	$6^{+}_{-}4$	$6^{+}_{-}4$

SUBJECT	Post Depletion				
	(1)	(2)	(2)-(1)	sIg	Facb
RA 1	3	3	0	<0.5	2
RA 2	3	5	2	<0.5	4
RA 3	3	3	0	<0.5	17
RA 4	3	3	0	<0.5	6
mean $\pm$ 1 S.D.	-	-	$1^{+}_{-}1$	<0.5	$7^{+}_{-}6$


Pre Depletion
Post Depletion
% Positive Cells
20
15
10
5
Anti light chain rosettes
sIg
Facb Rosettes

populations and sIg bearing cells were measured as described in Chapter 2, Section II (v). Loss of sIg bearing cells tended to increase Facb receptor positive cells which indicates that the Facb receptor is not present on B cells.

The results shown in figure 5.4 indicate that Facb receptor bearing cells are also C3 receptor negative. Mononuclear cell preparations were depleted of Facb rosettes and EA, Facb and C3 rosettes were prepared. Complement receptor positive cells were increased after Facb rosette depletion.

Styrene beads have been used for the selective depletion of B cells (Pang and Wilson, 1978). Columns of styrene beads were used for the separation of Ficoll-Paque prepared lymphocytes (Chapter 2, Section II (v)) from patients with RA, AS and from healthy subjects. In all cases total EA rosettes were decreased in the unbound fraction in comparison with the unfractionated mononuclear cell preparation (Fig. 5.5). Facb rosette forming cells were removed only from rheumatoid cells after passage through styrene beads.

(iii) Cell Lines

Many of the experiments described above utilised Facb rosette depletions. Facb rosettes seem to be very fragile and break up easily during handling. A number of cell lines were tested for Facb rosette formation since an Facb receptor positive cell would prove ideal for studies of receptor characterisation. Also antibody to isolated receptors could be coupled to cyanogen bromide activated Sepharose to facilitate removal of Facb receptor positive

Fig. 5.4

Facb ROSETTE DEPLETION OF MONONUCLEAR CELL PREPARATIONS : EFFECT ON COMPLEMENT RECEPTOR (C3R) POSITIVE CELLS

Mononuclear cell preparations (n = 4) were depleted of Facb rosetting cells. EA, Facb and C3 rosettes were prepared before and after depletion. A decrease in Facb rosette forming cells produced an increase in C3R positive cells. The increase did not reach significance but these results indicate that Facb rosette forming cells do not possess complement receptors. EA rosettes were significantly decreased.

	% ROSETTES $\pm$ 1 S.D.			
	TOTAL EA	HIGH AVIDITY EA	Facb	COMPLEMENT (C3)
Pre Facb Depletion	23 ± 2	10 ± 8	8 ± 5	6 ± 3
Post Facb Depletion	16 ± 2	3 ± 2	3 ± 1	13 ± 6
Significance (Student's t test)	$p < 0.01$	NS	NS	NS

NS = not significant

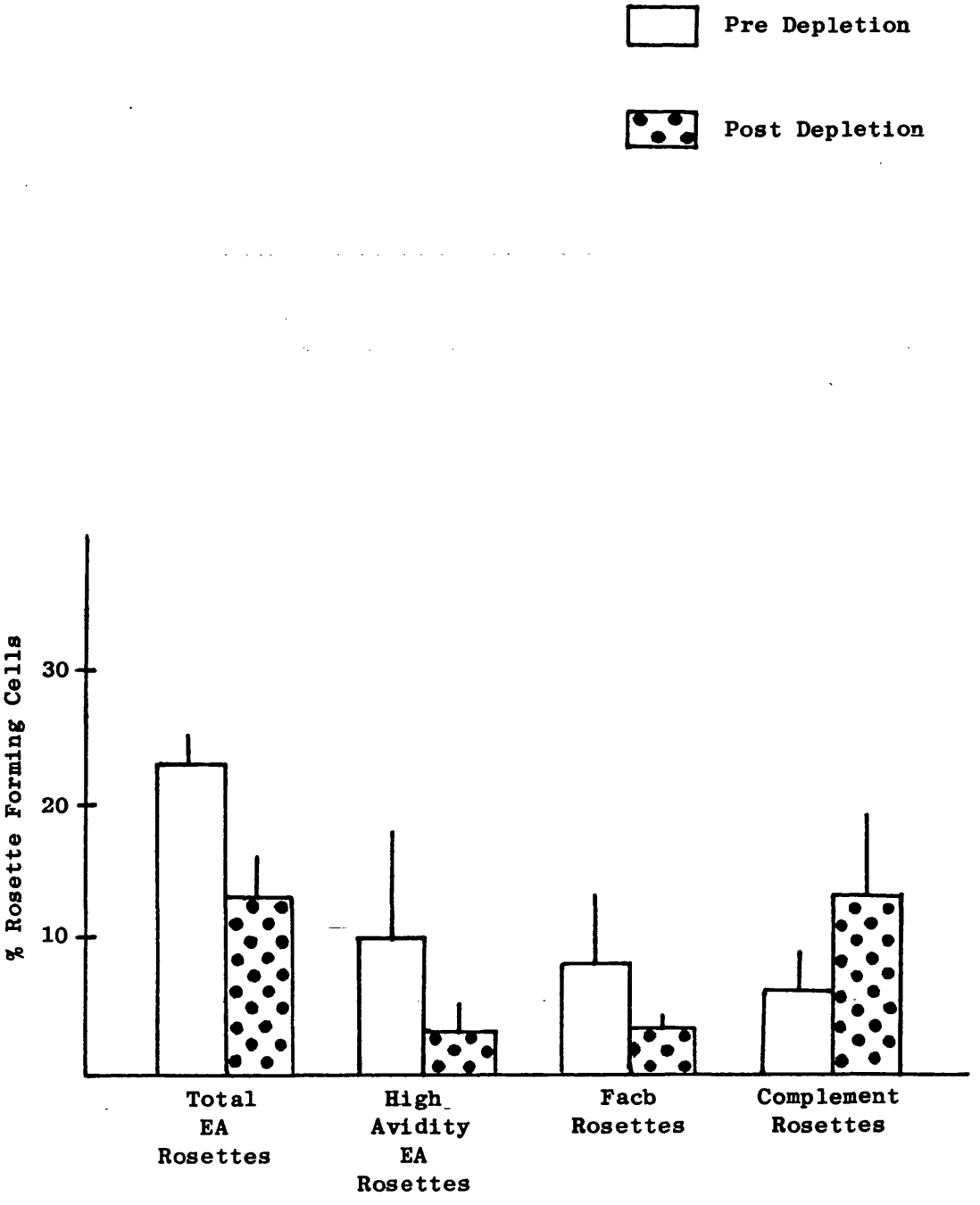

Pre Depletion
Post Depletion
% Rosette Forming Cells
30
20
10
Total EA Rosettes
High Avidity EA Rosettes
Facb Rosettes
Complement Rosettes

Fig. 5.5.

EFFECT OF STYRENE BEAD COLUMN CHROMATOGRAPHY ON LYMPHOCYTE SUBPOPULATIONS FROM PATIENTS WITH RHEUMATOID ARTHRITIS (RA) AND ANKYLOSING SPONDYLITIS (AS) AND HEALTHY INDIVIDUALS

The methodology is described in Chapter 2, Section II (v).

Legend

Unfractionated Cell Population

Non Adherent Population

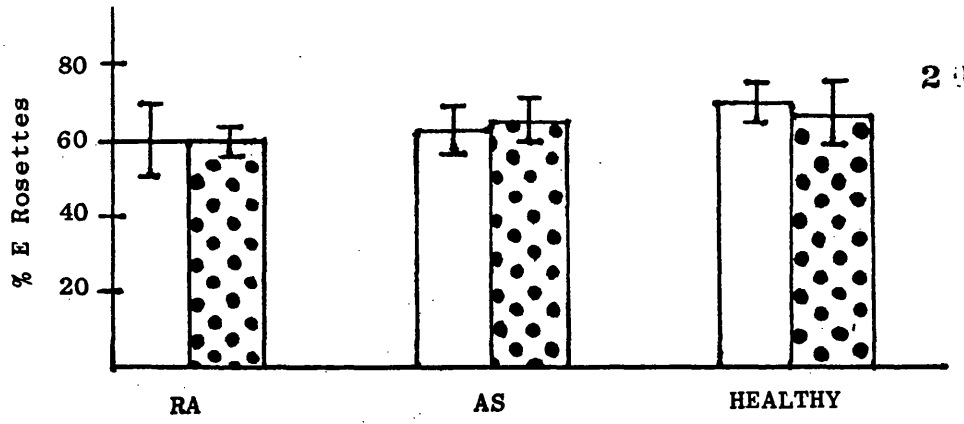
RESULTS

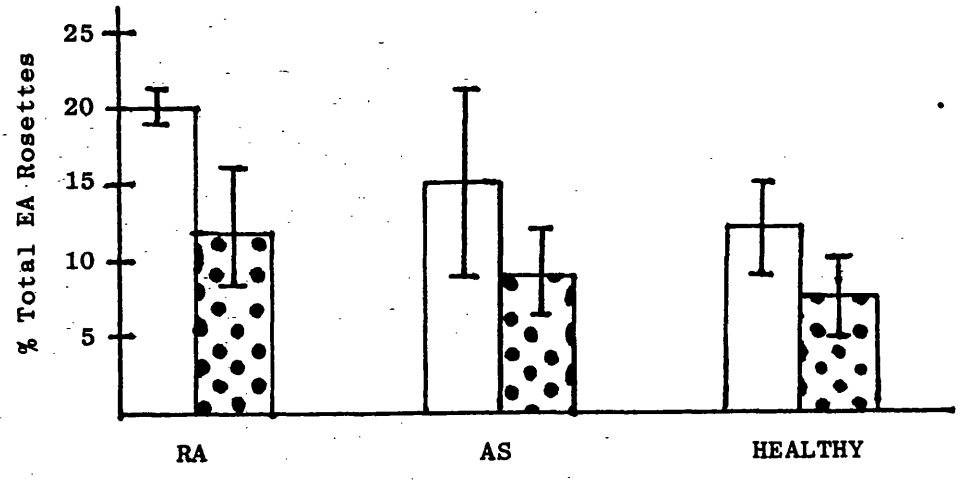
		% ROSETTES			
		E	TOTAL EA	HIGH AVIDITY EA	Facb
RA (n=5)	Unfractionated	$60^{+}_{-}10$	$20^{+}_{-}1$ **	$8^{+}_{-}4$	$10^{+}_{-}3$ **
	Non Adherent	$60^{+}_{-}4$	$12^{+}_{-}4$ **	$4^{+}_{-}2$	$1^{+}_{-}1$ **
AS (n=6)	Unfractionated	$61^{+}_{-}7$	$15^{+}_{-}6$	$5^{+}_{-}1$ **	$5^{+}_{-}2$
	Non Adherent	$64^{+}_{-}7$	$9^{+}_{-}3$	$2^{+}_{-}1$ **	$4^{+}_{-}1$
HEALTHY (n=5)	Unfractionated	$68^{+}_{-}5$	$12^{+}_{-}3$ *	$3^{+}_{-}2$	$2^{+}_{-}1$
	Non Adherent	$66^{+}_{-}10$	$7^{+}_{-}2$ *	$2^{+}_{-}1$	$1^{+}_{-}1$

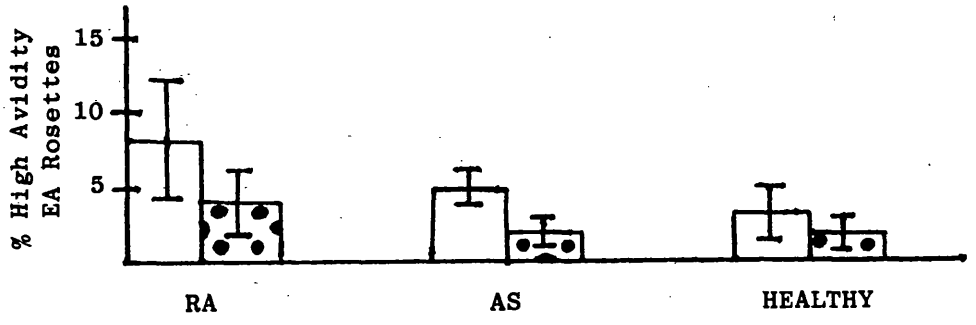
Significant Results (Student's t test) ** $p < 0.01$

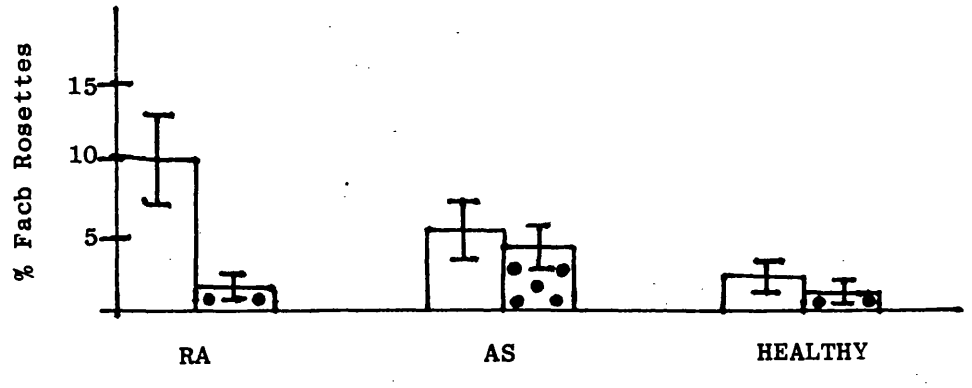
* $p < 0.05$

n = number of subjects tested


% E Rosettes
80
60
40
20
RA
AS
HEALTHY


% Total EA Rosettes
25
20
15
10
5
RA
AS
HEALTHY


% High Avidity EA Rosettes
15
10
5
RA
AS
HEALTHY


% Facb Rosettes
15
10
5
RA
AS
HEALTHY

cells. The myeloid leukaemia cell line K562 is known to possess a high density of $Fc_{\gamma}Rs$ (Klein et al, 1976). However, this and all the others tested proved to be Facb receptor negative (Table 5.6). In contrast, the $F(ab')_2$ fragment of an IgG antibody raised against isolated K562 Fc receptors (a gift from Dr. D. Hough, Biochemistry Department, University of Bath) inhibited both total EA and Facb rosettes prepared using rheumatoid peripheral blood mononuclear cells. Normal rabbit $F(ab')_2$ showed some inhibition but, in these experiments, did not reach significance (Fig. 5.6). A human FcR preparation, obtained by temperature shift experiments (Chapter 2, Section II (iv)) showed no significant inhibition of either high avidity EA or Facb rosettes in the five rheumatoid patients tested (Table 5.7); total EA rosettes were not tested. The supernatant containing shed receptors was not concentrated prior to use as a competitor.

Cell lines are often derived from leukaemic cells. During the course of these investigations on Facb receptors, two patients with leukaemias were tested. One individual with PA and monocytic leukaemia showed high percentage Facb rosettes (17%) while the second individual with OA of the hip and chronic lymphocytic leukaemia (CLL) possessed low percentages of peripheral blood Facb rosette forming cells ($< 1\%$).

(iv) Effect of Culture and Mitogenic Stimulation on the Expression of the Facb Receptor

FcR expression can be associated with cell activation. Peripheral blood mononuclear cells prepared from rheumatoid and healthy subjects were incubated overnight and with or without mitogens over a period

Table 5.6

Facb ROSETTE FORMATION BY VARIOUS CELL LINES

CELL LINE	SOURCE	% Facb ROSETTES (Mean $\pm$ 1 S.D.)	COMMENTS
K562	Human	6 ± 2	one line tested five times
Raji	Human	3 ± 2	one line tested five times
Leukaemia (L 1210)	Murine	1	one line tested once
EBV Transformed *	Human Healthy	3 ± 1	twelve lines tested once
EBV Transformed *	Human Rheumatoid	3 ± 1	six lines tested once

*Provided by Dr. A. B. Rickinson, Department of Pathology, University of Bristol

Table 5.7

INHIBITION OF RHEUMATOID PERIPHERAL BLOOD LYMPHOCYTE HIGH AVIDITY EA and Facb RECEPTORS BY A HUMAN Fc RECEPTOR PREPARATION OBTAINED BY TEMPERATURE SHIFT

	% ROSETTES	NO. TESTED
Facb	6 ± 2	5
Facb + 40 μl supernatant	7 ± 3	5
High Avidity EA (EA_{HA})	11 ± 4	5
EA_{HA} + 40 μl supernatant	13 ± 5	5

Fig. 5.6

SPECIFICITY OF K562 Fc RECEPTOR ANTIBODY FOR Facb RECEPTORS ON RHEUMATOID PERIPHERAL BLOOD MONONUCLEAR CELLS

The $F(ab')_2$ fragment of a rabbit IgG antibody raised against purified K562 Fc receptors was used as a competitor in the EA and Facb rosette assays at a concentration of 40 μg/ml. The $F(ab')_2$ fragment of normal rabbit IgG was also used at the same concentration.

Legend

(a) Total percentage rosettes (no competitor)
(b) $F(ab')_2$ fragment of rabbit anti K562 FcR added as competitor
(c) $F(ab')_2$ fragment of normal rabbit IgG added as competitor

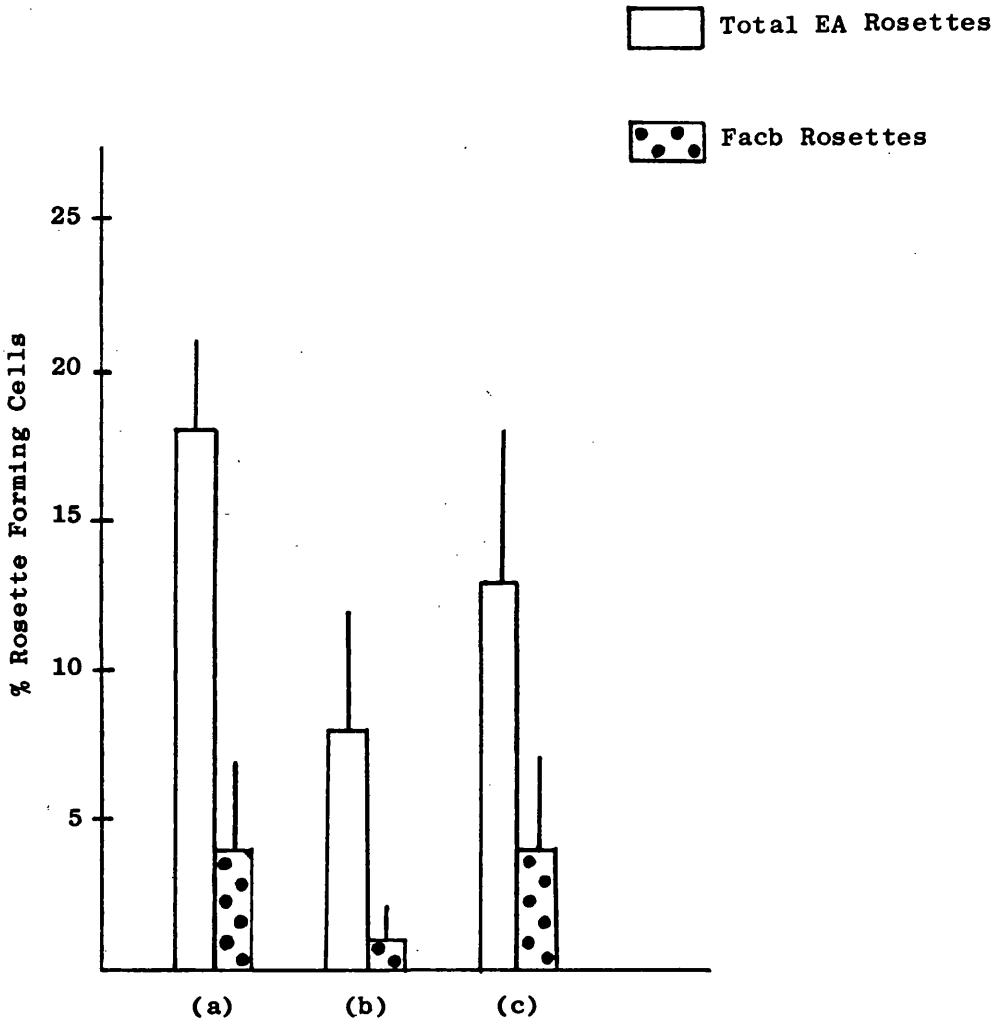
RESULTS

ADDITION (40 μg/ml)	ROSETTES (Mean $\pm$ 1 S.D.) TOTAL EA		Facb	
None (a)	18 ± 3 (n=4)	**	4 ± 3 (n=6)	*
$F(ab')_2$ anti K562 FcR (b)	8 ± 4 (n=4)		1 ± 1 (n=6)	
normal rabbit $F(ab')_2$ (c)	13 ± 5 (n=4)		4 ± 3 (n=4)	

n = number of subjects tested

Significant results (Student's t test)

** $p < 0.01$
* $p < 0.05$


Total EA Rosettes
Facb Rosettes
% Rosette Forming Cells
25
20
15
10
5
(a)
(b)
(c)

of 60-70 hours (Chapter 2, Section II (vi)). EA and Facb rosettes were prepared on all samples. The results are shown in figures 5.7 and 5.8. Incubation overnight did not significantly affect Facb receptor expression. However, longer incubations caused a decrease in percentage detectable Fc receptors using both rosette assays irrespective of whether cells were subjected to mitogenic stimulation or merely cultured without mitogen. Only concanavalin A stimulated healthy cells showed an insignificant percentage decrease of Facb receptor positive cells with respect to freshly prepared cells.

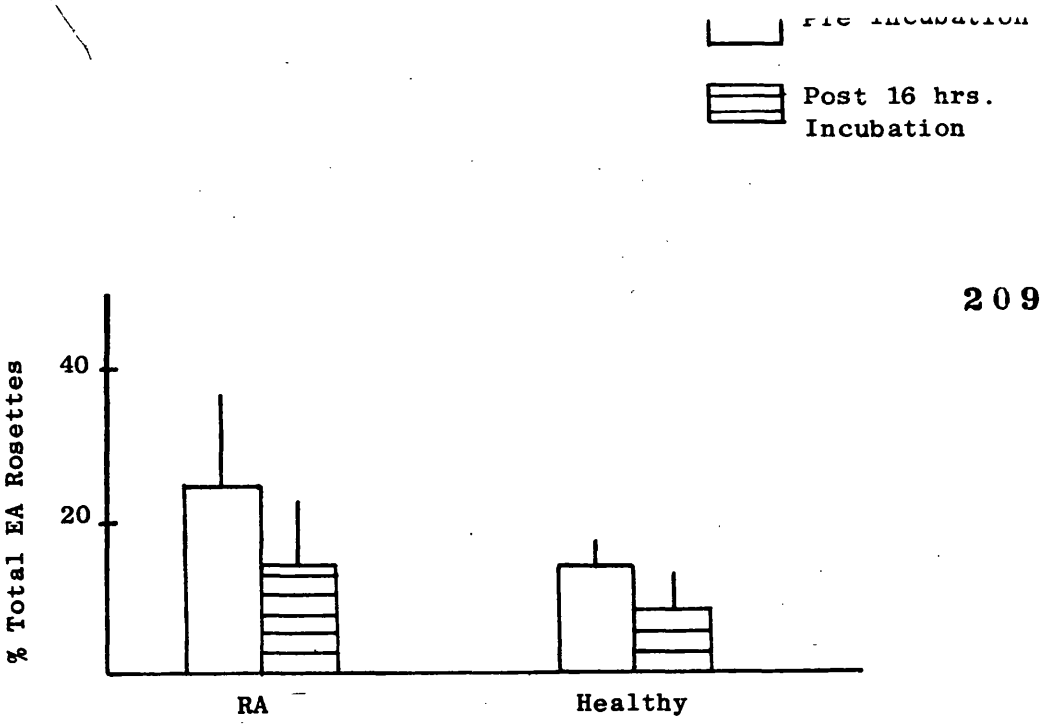
Fig. 5.7

EFFECT OF INCUBATION ON Facb RECEPTOR POSITIVE PERIPHERAL BLOOD CELLS

Peripheral blood mononuclear cells were incubated overnight (16 hours). Rosettes were prepared before and after incubation.

SUBJECTS	NO. TESTED		% ROSETTES (Mean ± 1 S.D.) TOTAL EA	Facb
RA	6	Pre incubation	25±11	10±6
		Post incubation	14±9	7±5
Healthy	6	Pre incubation	14±3 *	3±2
		Post incubation	8±5 *	3±2

Significant Results (Student's t test) * $p < 0.05$


Pre Incubation
Post 16 hrs. Incubation
% Total EA Rosettes
40
20
RA
Healthy

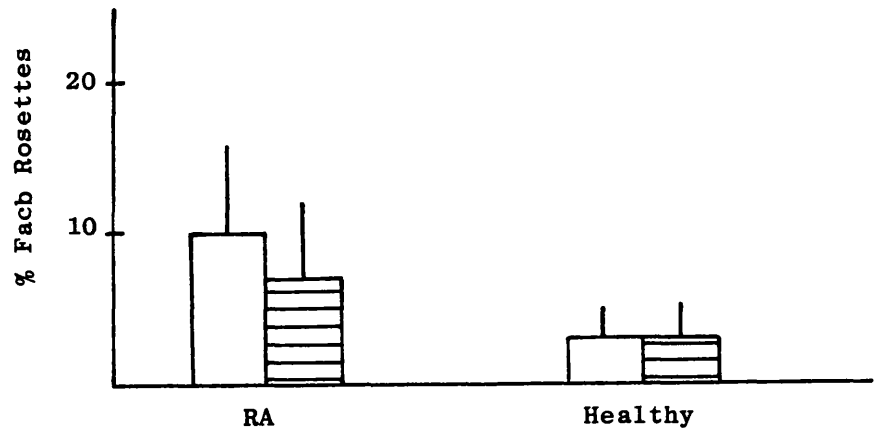

% Facb Rosettes
20
10
RA
Healthy

Fig. 5.8

EFFECT OF MITOGENIC STIMULATION ON Facb RECEPTOR EXPRESSION BY PERIPHERAL BLOOD CELLS

Peripheral blood mononuclear cells were incubated with or without mitogens for 60-70 hours. Percentage EA and Facb rosettes were enumerated before and after incubation.

Legend

(a) Pre incubation (mononuclear cell preparation)
(b) Post incubation (no mitogen)
(c) Post incubation with 1 μg/ml phytohaemagglutinin
(d) Post incubation with 50 μg/ml concanavalin A
(3) Post incubation with pokeweed mitogen (1/40 dilution)

RESULTS

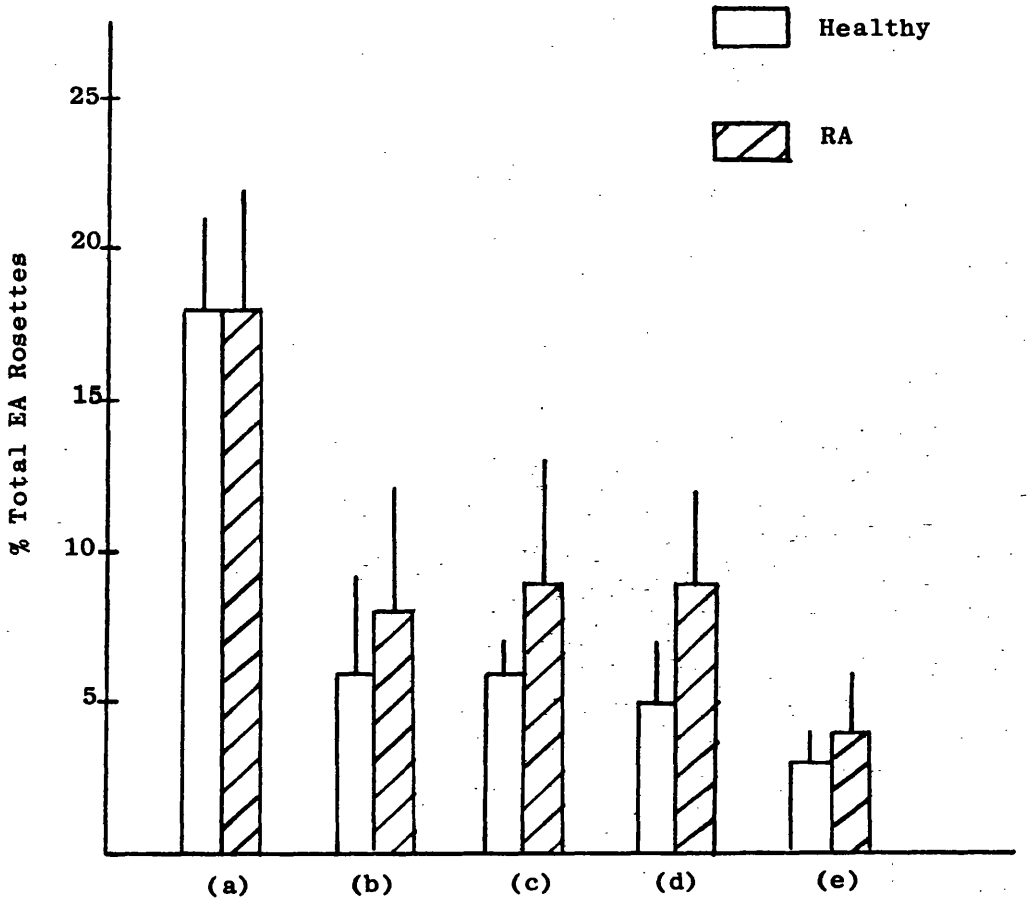
			% ROSETTES (Mean $\pm$ 1 S.D.)	
SUBJECT	SAMPLE	NO. TESTED	TOTAL EA	Facb
Healthy	(a)	3	18 ± 3 } **	7 ± 1 } *
	(b)	3	6 ± 3 }	2 ± 2 }
	(c)	3	6 ± 1	1 ± 1
	(d)	3	5 ± 2	5 ± 2
	(e)	3	3 ± 1	3 ± 2
RA	(a)	5	18 ± 4 } **	12 ± 3 } **
	(b)	5	8 ± 4 }	4 ± 2 }
	(c)	5	9 ± 4	4 ± 3
	(d)	5	9 ± 3	4 ± 3
	(e)	4	4 ± 2	4 ± 3

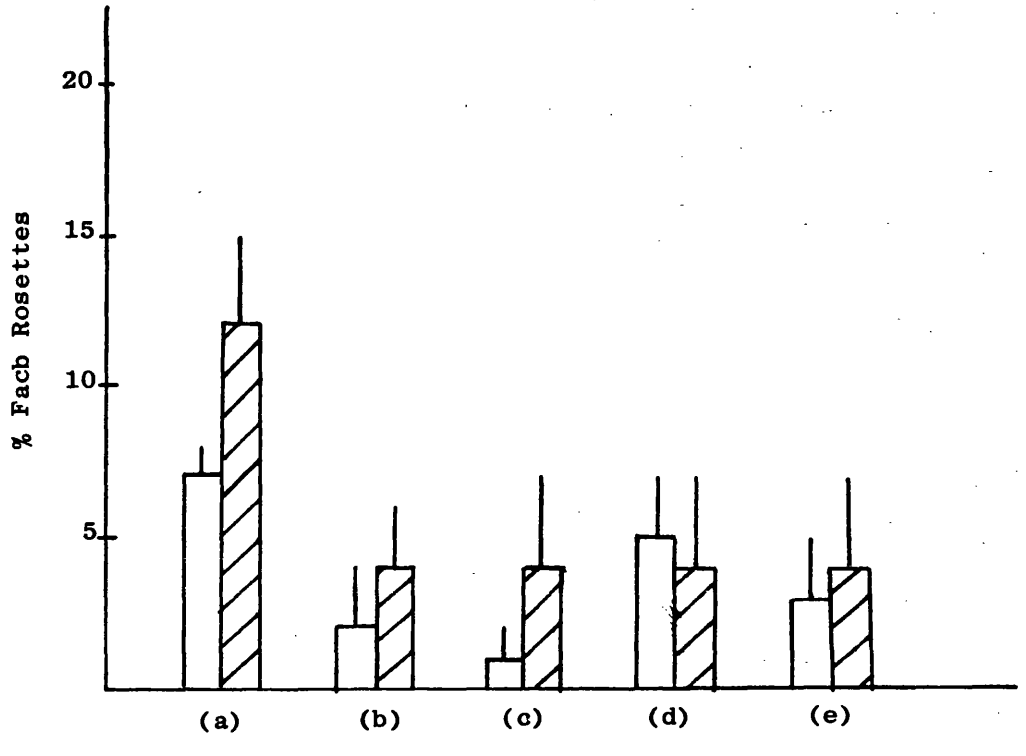
Significant Results (Student's t test)

** $p < 0.01$

* $p < 0.02$

RA Facb (a) Vs Healthy Facb (a) $p < 0.05$


Healthy
RA
% Total EA Rosettes
25
20
15
10
5
(a)
(b)
(c)
(d)
(e)


% Facb Rosettes
20
15
10
5
(a)
(b)
(c)
(d)
(e)

CHAPTER SIX

FUNCTIONAL ASPECTS OF F_{ACB} RECEPTOR POSITIVE LYMPHOCYTES

S E C T I O N I

INTRODUCTION

The results given in Chapters 4 and 5 have provided evidence for the specificity of the Facb receptor and the type of peripheral blood cell on which such receptors are detected. In Chapter 3, it was shown that Facb receptor positive peripheral blood cells are present in all individuals and that raised percentages occur in patients with RA and occasionally in healthy persons; skin testing of sensitised individuals also resulted in raised percentages of Facb rosette forming cells. This suggests that these cells may be important in the immune response to antigen. Functional aspects of FcR positive cells were reviewed in Chapter 1, Sections II and IV (iii). In this chapter, preliminary experiments will be described which attempt to ascribe a function to Facb receptor positive cells.

S E C T I O N I I

CYTOTOXICITY ASSAYS

Natural killer (NK) and killer (K) cells both bear $Fc_{\gamma}R$. Mononuclear cell populations were depleted of Fc receptor bearing cells and assessed for their ability to lyse chromium labelled (^{51}Cr) K562 target cells in the presence and absence of antibody.

(i) NK Cell Assay

The methodology is given in Chapter 2, Section II (vii). Some problems were experienced using the 4 hour assay described by Pope,

Moretta, Troy and Perlmann (1979) since the values obtained for specific release were very low (< 10%). The time taken to deplete rosettes and the fact that the cells undergo two passages through Ficoll-Paque may explain the low specific releases obtained. Mononuclear cells were prepared and an NK cell assay was set up at hourly intervals for three hours (Table 6.1; Assays I, II, III). The cell preparations were maintained at ambient temperature for the three hours. A fourth assay (Table 6.1; Assay IV) was set up at the same time as the final 4 hour assay (Table 6.1; Assay III) and incubated overnight. All of the 4 hour assays gave low specific releases and varied little with time. The 16 hour assay showed higher specific releases and was thus favoured for routine use. Table 6.2 shows the results obtained for 5 rheumatoid and 3 healthy individuals using the 16 hour assay. There was no significant difference between isolated peripheral blood rheumatoid and healthy lymphocytes in their ability to lyse K562 target cells in the absence of antibody.

The results obtained for NK cell activity following depletion of $Fc_{\gamma}R$ bearing cells are given in Table 6.3. All depletions were checked by re-rosetting. Sham depletions had no effect on natural killer cell activity. Therefore either undepleted or sham depleted populations were ascribed 100% NK activity and depleted populations were related to this figure. The results are presented diagrammatically in Figure 6.1. Although the numbers tested were small, it can be seen that total EA rosette depletion reduced natural killer cell activity indicating that $Fc_{\gamma}R$ positive cells are important in natural cytotoxicity. Depletion of either Facb receptor positive cells or high avidity EA rosette forming cells showed little effect

Table 6.1

NATURAL KILLER CELL ASSAY

COMPARISON OF TWO INCUBATION PERIODS

ASSAY NO.	INCUBATION TIME (hrs)	SPECIFIC ^{51}Cr RELEASE (%)			
		RA 1	RA 2	RA 3	HEALTHY
I	4	0.1	3.8	5.9	6.6
II	4	2.5	4.3	6.8	9.8
III	4	3.7	7.4	10.9	8.1
IV	16	18.4	9.6	24.8	24.3

Table 6.2

NATURAL KILLER CELL ASSAY

SUBJECT	INCUBATION TIME (hrs)	SPECIFIC ^{51}Cr RELEASE (%)	MEAN SPECIFIC ^{51}Cr RELEASE (%)
RA 1	16	18.4	
RA 2	16	9.6	
RA 3	16	24.8	20.0 ± 7.6
RA 4	16	29.9	
RA 5	16	12.3	
HEALTHY 1	16	24.3	
HEALTHY 2	16	34.1	25.7 ± 6.3
HEALTHY 3	16	18.8	

Table 6.3

NATURAL KILLER CELL ASSAY

EFFECT OF REMOVAL OF $Fc_{\gamma}R$ BEARING CELLS

MONONUCLEAR CELL PREPARATION	LYMPHOCYTE TO TARGET CELL RATIO	SPECIFIC ^{51}Cr RELEASE (%)					
		RA			HEALTHY		
		1	2	3	1	2	3
Pre Depletion	60:1 *	-	29.9	12.2	-	42.7	18.8
Sham Depleted	10:1	12.0	-	-	8.8	-	-
	60:1 **	-	18.5	13.1	-	67.4	19.4
Total EA Rosette Depleted	60:1	-	9.9	8.9	-	44.6	16.2
High Avidity EA Rosette Depleted	10:1	9.2	-	-	8.8	-	-
Facb Rosette Depleted	10:1	11.6	-	-	12.2	-	-
	60:1	-	36.8	7.1	-	63.6	29.8

* Mean specific ^{51}Cr release (%) = 25.9 $\pm$ 11.6 (n=4)

** Mean specific ^{51}Cr release (%) = 29.6 $\pm$ 22.0 (n=4)

There is no significant difference between the two samples

Fig. 6.1

NATURAL KILLER CELL ASSAY

EFFECT OF DEPLETION OF $Fc_\gamma R$ BEARING CELLS

The cytotoxicity assay was carried out as described in Chapter 2, Section II (vii). Mononuclear cell preparations were depleted of various $Fc_\gamma R$ positive subpopulations. The percentage cytotoxicity and Facb rosettes were calculated relative to the untreated or sham depleted population. The results are shown opposite. The columns and bars represent the mean cytotoxicity $\pm$ 1 standard deviation.

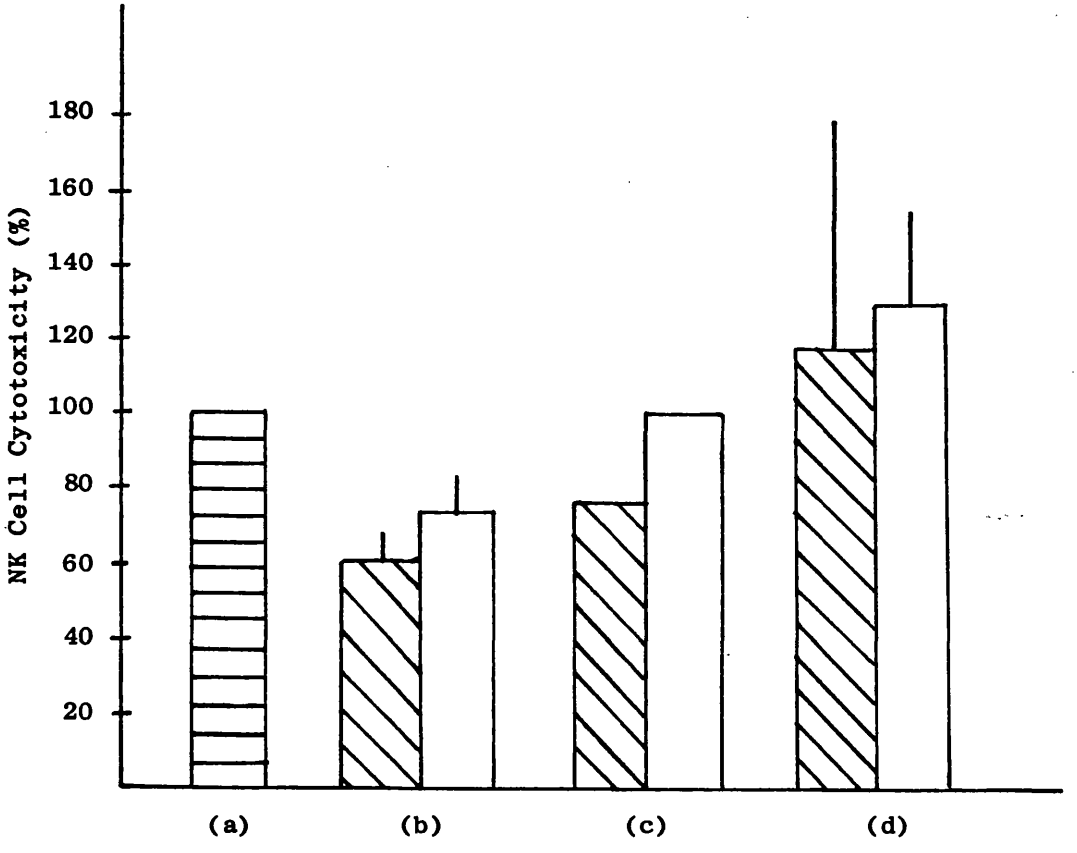
Results

MONONUCLEAR CELL POPULATION	SUBJECTS TESTED	NO. TESTED	% Facb ROSETTES	NK CELL CYTOTOXICITY (%)
Undepleted/Sham Depleted	RA	3	100	100
	HEALTHY	3	100	100
Total EA Rosette Depleted	RA	2	-	61 ± 7
	HEALTHY	2	-	75 ± 9
High Avidity EA Rosette Depleted	RA	1	-	77
	HEALTHY	1	-	100
Facb Rosette Depleted	RA	3	70 ± 60	117 ± 61
	HEALTHY	3	71 ± 22	129 ± 25

RA

Healthy

/(a) Sham Depleted
(b) Total EA Rosette Depleted
(c) High Avidity EA Rosette Depleted
(d) Facb Rosette Depleted



on NK cell activity. No differences in activity between rheumatoid or healthy individuals was apparent. Some enhancement of NK cell activity occurred following Facb rosette depletion indicating possible enrichment of NK cells. Thus, Facb receptor positive cells do not appear to mediate spontaneous cell mediated cytotoxicity.

(ii) K Cell Assay

The effects of depletion of $Fc_{\gamma}R$ bearing cells on K cell activity are shown in Table 6.4 and Fig. 6.2. The results in Fig. 6.2 are expressed relative to undepleted or sham depleted populations as described for the NK cell assay. Owing to the low cell yields obtained following Facb rosette depletion, low lymphocyte to target cell ratios were used in some instances. Depleted populations were checked by re-rosetting. Total EA rosette depletion drastically reduced K cell activity demonstrating a requirement for $Fc_{\gamma}R$ positive cells in antibody dependent cytotoxicity. However depletion of both Facb receptor positive cells and high avidity EA rosette forming cells produced an enhancement of K cell activity. Thus K cells do not appear to possess Facb receptors.

S E C T I O N I I I

LYMPHOCYTE ACTIVATION BY MITOGENS : EFFECT OF REMOVAL OF $Fc_{\gamma}R$ POSITIVE CELLS

FcR positive cells have been implicated in the response to mitogenic stimulation (Chapter 1, Section II and IV (iii)). Depletion experiments were carried out and the untreated and depleted populations reacted with optimal mitogen concentrations as described in Chapter 2,

Table 6.4

KILLER CELL ASSAY

EFFECT OF REMOVAL OF $Fc_\gamma R$ BEARING CELLS

MONONUCLEAR CELL PREPARATION	LYMPHOCYTE TO TARGET CELL RATIO	SPECIFIC ^{51}Cr RELEASE (%)						
		RA						HEALTHY
		1	2	3	4	5	6	
Pre Depletion (Carbonyl Iron Treated)	30:1	31.9	36.7	36.0				
Sham Depleted	5:1				12.3	12.0	13.9	12.0
Total EA Rosette Depleted	30:1 5:1	16.9	11.5	18.6	1.4	1.8		
High Avidity EA Rosette Depleted	5:1						18.2	13.1
Facb Rosette Depleted	30:1 5:1	36.1	33.9	34.0	13.9	10.5	18.0	14.8

Fig. 6.2

KILLER CELL ASSAY

EFFECT OF DEPLETION OF $Fc_{\gamma}R$ BEARING CELLS

Antibody dependent cytotoxicity was assayed as described in Chapter 2, Section II (vii). Mononuclear cell preparations were depleted of various $Fc_{\gamma}R$ positive subpopulations. The percentage cytotoxicity and Facb rosettes were calculated relative to the untreated or sham depleted population. The results are shown opposite. The columns and bars represent the mean cytotoxicity $\pm$ 1 standard deviation.

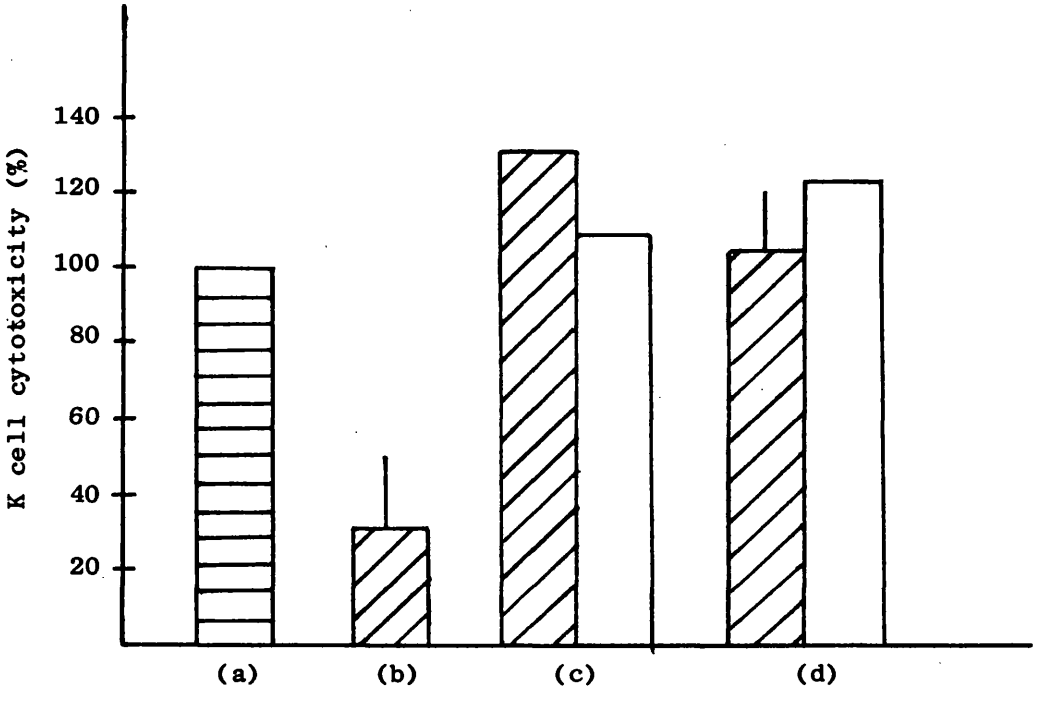
Results

MONONUCLEAR CELL POPULATION	SUBJECTS TESTED	NO. TESTED	% Facb ROSETTES	NK CELL CYTOTOXICITY (%)
Undepleted/Sham Depleted	RA	6	100	100
	HEALTHY	1	100	100
Total EA Rosette Depleted	RA	5	-	32 $\pm$ 18
	HEALTHY	-	-	
High Avidity EA Rosette Depleted	RA	1	-	131
	HEALTHY	1	-	109
Facb Rosette Depleted	RA	6	34 $\pm$ 5	105 $\pm$ 15
	HEALTHY	1	60	123

RA

Healthy

/(a) Sham Depleted
(b) Total EA Rosette Depleted
(c) High Avidity EA Rosette Depleted
(d) Facb Rosette Depleted



Section II (vi). The results are presented in Table 6.5. In many instances, sham depletions affected mitogen responses and little difference occurred between $Fc_{\gamma}R$ positive cell depleted and sham depleted populations. Occasional samples showed increased or decreased stimulation but no simple pattern of effects is apparent.

Table 6.5

LYMPHOCYTE ACTIVATION BY MITOGENS

EFFECT OF REMOVAL OF $Fc_{\gamma}R$ POSITIVE CELLS

MONONUCLEAR CELL PREPARATION	MITOGEN ADDED	STIMULATION INDEX							
		HEALTHY				RA			
		1	2	3	4	1	2	3	4
Pre Depletion	PHA	82	12	6	2	1	1	2	31
	Con A	21	10	5	19	1	1	1	8
	PWM	24	8	6	8	2	1	2	8
Sham Depleted	PHA	8	20	-	4	1	1	2	7
	Con A	5	12	-	4	5	1	16	2
	PWM	4	6	-	7	2	1	3	2
Total EA Rosette Depleted	PHA	7	-	9	-	-	-	-	18
	Con A	2	-	7	-	-	-	-	4
	PWM	8	-	3	-	-	-	-	9
Facb Rosette Depleted	PHA	9	28	2	5	1	1	5	12
	Con A	2	13	1	12	2	1	3	4
	PWM	3	6	2	5	1	2	3	4

$$\text{STIMULATION INDEX} = \frac{\text{mean cpm of stimulated cells}}{\text{mean cpm of unstimulated cells}}$$

CHAPTER 7

DISCUSSION

Facb Receptor Positive Cells

(i) Surface Characterisation and Relationship to Null Lymphocytes

Receptors for the Fc portion of immunoglobulin (FcRs) are present on some subsets of all human peripheral blood leucocytes (Chapter 1, Section III (i)). In this thesis, a rosette assay has been described which detects an FcγR binding the C_H2 region of IgG (Facb receptors). The characterisation of the lymphocyte bearing Facb receptors was described in Chapter 5. Such cells were shown to lack receptors for Helix pomatia lectin, sheep erythrocytes and complement (C3) and were surface Ig negative. Thus the Facb receptor (FacbR) positive lymphocyte is a C3 receptor negative null lymphocyte. Null lymphocytes have been described as third population cells (Frøland, Wisløff and Michaelsen, 1974), L cells (Horwitz and Lobo, 1975) and UL cells (Dickler, 1976 b). Pang and Wilson (1978) showed that null cells formed EA rosettes at low concentrations of sensitising antibody. Similar results were obtained for FacbR positive lymphocytes (Fig. 3.4 and Fig. 4.1). Cells forming EA rosettes at low antibody concentrations will be termed high avidity EA rosettes.

L lymphocytes are C3 receptor negative non-T non-B lymphocytes; these cells have been shown to react with anti-myeloid sera (Horwitz, Niaudet, Greaves, Dorling and Deteix, 1978). This is an interesting observation since a high percentage of Facb rosettes (17%) was detected in a subject with monocytic leukaemia (Chapter 5, Section III (iii)) but this patient also had RA. The myeloid leukaemia cell line K562 did not bind Facb sensitised erythrocytes although a rabbit IgG antibody raised against isolated K562 FcRs inhibited Facb and

total EA rosette formation (Chapter 5, Section III (iii)). The meaning of these conflicting results is unclear. It is possible that the rabbit antiglobulin is raised against a FcR componenet which is normally inaccessible on the intact cell but is exposed following receptor isolation; it is known that enzymes (e.g. neuraminidase) can effect receptor expression (Hammarstrøm et al, 1973). However if the component is normally hidden it would seem unlikely that the antibody could block EA rosette formation.

FacbR bearing lymphocytes do not appear to be myeloid since removal of cells staining with non-specific esterase did not decrease the percentage of detectable Facb rosettes (Table 5.3). However, Kay and Horwitz (1980) have shown reactivity of the monoclonal antibody OKM1, which is thought to detect myeloid/monocytic cells, with L lymphocytes; a previous report had shown L lymphocytes to be non phagocytic and esterase negative (Niaudet, Greaves and Horwitz, 1979). The recent production of the monoclonal antibodies has further complicated the detection of lymphocyte subpopulations since OKM1 positive cells are found not only in the null cell fraction but also in the Tγ population (Reinherz et al, 1980), and some Tγ cells have been shown to contain non-specific esterase (Manconi et al, 1979). It is possible that the use of neuraminidase treated sheep erythrocytes (SRBC) in the isolation of Tγ cells may lead to a loss of specificity for T cells. FacbR bearing cells did not seem to react with neuraminidase treated SRBC since E rosette depletion enhanced the percentage detectable Facb rosettes (Fig. 5.1).

It was thought that the FacbR positive lymphocyte might be an immature cell but incubation of isolated peripheral blood mono-

nuclear cell populations for periods up to 70 hrs resulted in a loss of FcRs, and mitogenic stimulation did not enhance FacbR expression (Figs. 5.7 and 5.8). Immature cells show increased FcR expression with maturation (Yang et al, 1978) while blast transformed cells lack FcRs (Jondal, 1974).

Ripley rosettes have been shown by some workers to detect cells other than third population cells (Doblougeet al, 1979; Andersson and Svennevig, 1980). Likewise, Facb rosettes were shown to detect FacbRs on some polymorphonuclear leucocytes and monocytes (Table 5.4 and 5.5). None of the cell lines tested expressed high percentages of Facb receptors (Table 5.6). As previously stated, the sensitising antibody was not titrated for detection of polymorphonuclear leucocyte or monocyte FcRs and therefore, the percentage total number of FcγR and FacbR positive cells may be higher.

It thus appears that FacbR positive lymphocytes show characteristics of null lymphocytes and may be analogous to the L cell population or form a subset of L cells.

(ii) Binding Specificity of the Facb Receptor

Third population cells have been shown to preferentially bind IgG1 and IgG3 (Frøland et al, 1974) and L cells have been shown to form Ripley rosettes (Horwitz, 1977). Similarly, FacbR positive C3R negative null cells showed strong subclass specificity for IgG1 and IgG3 although IgG4 showed some binding (Fig. 4.2(a)). Monocytes also show subclass preference for IgG1 and IgG3

(MacLennan, 1972; Lawrence, Weigle and Spiegelberg, 1975; Dorrington, 1977) while neutrophils bind IgGs 1, 3 and 4 (Lawrence, Weigle and Spiegelberg, 1975). It is apparent that all FcγRs seem to exhibit G1, 3 specificity and this may partly explain why the Facb rosette assay detects some receptors on monocytes and neutrophils.

High avidity EA rosette forming cells and FacbR positive cells showed identical human IgG subclass specificity (Fig. 4.2 (a)). However fragments of rabbit IgG showed differential binding between the two cell populations. FacbR positive cells did not bind the C_H3 domain (pFc' fragment) of rabbit IgG but showed some binding of $F(ab')_2$ fragments while high avidity EA rosettes were not inhibited by the addition of $F(ab')_2$ fragments (Fig. 4.2 b). This implies that the hinge region is possibly involved in FacbR binding. It has been showed that Ripley rosette formation is not inhibited by pFc', which agrees with the finding for FacbR positive cells; however, using Ripley rosettes, $F(ab')_2$ showed no inhibition of binding and the Fc fragment was strongly inhibitory (Frøland et al, 1974). Both Facb and high avidity EA rosettes were inhibited by Fc fragments in this study, but the inhibition was less marked using Facb rosettes. Frøland and co-workers (1974) concluded that third population lymphocyte FcγRs bind primarily the C_H2 region of IgG. This is in agreement with the results using FacbR positive cells although, in this case, binding may also involve the hinge region. Other workers have suggested stabilisation of one binding site (C_H2) by a second (C_H3) (Ovary, Saluk, Quijada and Lamm, 1976) or co-operative binding through quarternary structure (Spiegelberg et al, 1975; Ovary et al, 1976). Binding to FacbRs may involve some interaction

at the hinge region, possibly an altered structure due to antigen binding, and immune complex formation.

Some monocytes express FacbRs (Table 5.5); Ciccimara, Rosen and Merler (1975) showed that the primary site of attachment of IgG to monocyte FcRs was in the C_H3 domain. Perhaps the altered hinge, after immune complex formation for example, exposes another binding site on the C_H2 region of the immunoglobulin molecule. However, a more likely explanation is that a few monocytes have a higher Fc receptor density and are therefore able to bind Facb. Another possibility is that the observed monocyte binding is merely due to contaminating lymphocytes or neutrophils. If the FacbR is an immune complex receptor, one might expect to find high percentages of FacbR positive neutrophils; this is not the case (Table 5.4).

Receptors shed as a result of a temperature shift from $4^{o}C$ to $37^{o}C$ did not inhibit Facb or high avidity EA rosettes (Table 5.7). Inhibition of total EA rosettes was not assayed. It has also been shown that Facb receptors are not shed as a result of temperature shift experiments (L.J.Eales - unpublished observations).

The competition results suggest that the FacbR positive cell and the high avidity EA rosette forming cell show some heterogeneity in their FcγRs, although there is no clear evidence to support the existence of two different cells or two different receptors on the same cell.

(iii) FacbRs - Susceptibility to Enzyme Treatment

Third population cells and L lymphocytes have been shown to possess trypsin resistant, pronase sensitive FcγRs while B cells have trypsin sensitive FcγRs (Lobo and Horwitz, 1976; Winfield, Lobo and Hamilton, 1977). Healthy FacbR positive cells were also found to be trypsin resistant (Fig. 4.4) which further strengthens the argument that these cells are not B cells but belong to the null cell population. Neuraminidase enhanced FacbR expression (Fig. 4.5); this is probably a surface charge related phenomenon. The increased percentage E rosettes observed using neuraminidase treated sheep erythrocytes is due to loss of negative surface charge by removal of sialic acid residues (Bentwich, Douglas, Skutelsky and Kunkel, 1973); a similar effect may occur with Facb rosettes. Competition experiments using saccharide molecules showed that most of the sugars used increased Facb rosette formation and decreased high avidity EA rosette formation. The acetyl hexosamine, N-acetyl-D-galactosamine (NADG), enhanced rosette formation in both assays (Fig. 4.3) but none of the results reached significance. One could speculate that this sugar may be a constituent of the binding site itself. The addition of NADG may cause membrane changes resulting in increased FacbR expression or a change in surface charge or may increase accessibility of FacbR; it is known that NADG has membrane effects since it inhibits the early phospholipid response to PHA in that there is a decreased incorporation of phosphatidyl choline (Fisher and Mueller, 1969).

Phospholipase C had no effect on the expression of FacbRs by healthy cells; however percentage total EA rosettes were sensitive

to low concentrations of this enzyme and the percentage of detectable cells fell to the level of percentage FacbR positive cells. Higher concentrations of phospholipase C produce no further decrease in detectable total EA rosette forming cells (Fig. 4.6). This is interesting since it has been shown that the FcγR is isolated from B lymphoblastoid cells has bound phospholipid and exhibits phospholipase A_2 activity (Suzuki, 1981). The FacbR appears not to have bound phospholipid but a proportion of the total EA rosette forming cell population was sensitive to phospholipase C.

The results obtained for total EA rosette agree with those described by Wilkinson (1977). The Facb receptor thus appears to be trypsin, phospholipase C and neuraminidase resistant. Phospholipase C hydrolyses only the polar head groups of several phospholipids including phosphatidylchōline and sphingomyelin. Wilkinson (1977) showed almost complete inhibition of EA rosette formation and leucocyte chemotaxis by the addition of sphingomyelinase C; this enzyme has a specificity for sphingomyelin only. Wilkinson (1977) suggests that the tail region of sphingomyelin may be important for cell interactions with extrinsic molecules. It is therefore possible that FacbRs have membrane associated sphingomyelin but no direct experiments have been performed.

(iv) Functional Aspects of FacbR Positive Lymphocytes

Both null and Tγ cells have been shown to exhibit K cell activity (Wisløff, Frøland and Michaelsen, 1973; Horwitz and Garrett, 1977; Gupta, Fernandes, Nair and Good, 1978). Kay and Horwitz (1980)

showed that all null and Tγ cells which effected ADCC reacted with the monoclonal antibody OKM1 and thus may be of myeloid origin. FacbR positive lymphocytes, however, showed no K cell activity; removal of Facb rosette forming cells increased K cell activity (Fig. 6.2). Null and Tγ OKM1 positive cells have also been shown to exhibit NK cell activity (Horwitz and Garrett, 1977; Gupta, Fernandes, Nair and Good, 1978; Kay and Horwitz, 1980). Facb rosette depleted mononuclear cell populations had normal or increased SCMC activity (Fig. 6.1). Removal of total EA rosette forming cells significantly reduced both ADCC and SCMC confirming the requirement for FcγR positive cells. However removal of cells with high avidity FcγRs, defined by the criteria described in Chapter 3, Section ii, failed to remove cytotoxic activity in all cases except one (Fig. 6.1). Further studies are necessary to verify this result. It is generally accepted that cytotoxic cells (K and NK) bear high avidity FcγRs.

The effect of depletion of populations of FcγR bearing cells on mitogenic stimulation is presented in Table 6.5. The results are inconclusive in that no pattern of reactivity is apparent. Sham depletions using unsensitised calf cells affected mitogenic stimulation. Stimulation indices dropped, rose or remained unchanged. It is possible that the introduction of foreign antigen (calf erythrocytes) activates the lymphocytes thereby lowering the observed stimulation index. However this is unlikely as the unstimulated cells did not show high background tritiated thymidine incorporation. It is more likely that the second passage through Ficoll-Paque removed most of the monocytes which may be important in this system. It is possible that the use of pre-determined optimal mitogen concentrations

affected the results. However, owing to the low yields obtained following rosette formation and two passages through Ficoll-Paque, it was only possible to use one concentration for each mitogen and to test for rosette depletion by re-rosetting.

Although the numbers of individuals used in the cytotoxicity assays are low, it is apparent that FacbR positive cells do not have cytotoxic potential. In contrast, L cells and third population cells have been shown to lyse antibody coated target cells (Horwitz and Garrett, 1977; Wisløff, Frøland and Michaelsen, 1974). It is possible that FacbR positive cells form a subset of L cells which do not have cytotoxic potential. The mitogen experiments provided no clue to their possible helper or suppressor function.

(v) Facb Receptor Positive Lymphocytes in Rheumatoid Arthritis

A clear difference observed between rheumatoid and healthy mononuclear cells was the significantly raised percentage numbers of FcγR and FacbR positive cells (Figs. 3.2 and 3.3). The raised percentage of EA rosettes is in agreement with the findings of other workers (Bach, Delrieu and Delbarre, 1970; Sharpin and Wilson, 1977 a; Wooley and Panayi, 1978). Evidence provided in this thesis concerning FacbR positive cells describes them as non-B non-T lymphocytes. However both third population and L lymphocytes have been reported in normal percentages in rheumatoid peripheral blood (Frøland, Natvig and Wisløff, 1975; Horwitz and Juul-Nielsen, 1977) although Horwitz and Juul-Nielsen (1977) found that absolute numbers of L cells were depressed. FacbR positive cells are present in

very low percentages in healthy peripheral blood (Fig. 3.5) while percentage L or third population cells are much higher (10-30% Frøland, Natvig and Wisløff, 1975; Horwitz and Juul-Nielsen, 1977; Lobo, 1981). It is possible that FacbR positive cells constitute a subpopulation of null (L) cells, or alternatively that the FacbR is only expressed following molecular or cellular interaction. However incubation or mitogenic stimulation of mononuclear cells failed to increase the percentage number of detectable FacbR bearing cells (Figs. 5.7 and 5.8). The increased binding of Facb sensitised erythrocytes by rheumatoid lymphocytes could possibly be artefactual since Alexander, Titus and Segal (1978) have described binding of serum IgG to ficoll/hypaque prepared lymphocytes. Pre-washing of whole blood only served to increase the percentages of detectable FacbR bearing cells in rheumatoid peripheral blood (Fig. 3.13). Trypsin also significantly increased the percentage of detectable Facb rosettes in rheumatoid peripheral blood (Fig. 4.4). Both these observations probably reflect the removal of immune complexes or IgG from previously blocked receptors. Binding studies have suggested possible immune complex binding by FacbRs (discussed previously in this chapter). Synovial fluid and peripheral blood contained similar percentage numbers of FacbR positive lymphocytes. This is in agreement with Abrahamsen (1981) who showed similar percentages of third population lymphocytes in rheumatoid peripheral blood and synovial fluid.

Rheumatoid lymphocyte cell membranes appear to have different properties than those of healthy lymphocytes. Phospholipase C treatment of rheumatoid lymphocytes produced a concentration dependent

decrease of total EA rosettes (Fig. 4.6). This may reflect an increased or altered lipid content of rheumatoid membranes. Rheumatoid Facb rosette forming cells were phospholipase C resistant as shown by healthy cells. FacbR positive rheumatoid lymphocytes differed from those of healthy subjects and patients with ankylosing spondylitis in their affinity for styrene beads. Only rheumatoid FacbR bearing lymphocytes adhered to columns of styrene beads; this implies that the cells are more "sticky" and this may explain why Horwitz and Juul-Neilsen (1977) found no differences between rheumatoids and controls in terms of cell numbers since these authors employ an adherence step to deplete monocytes. Frøland, Natvig and Wisløff (1975) observed depletion of third population rheumatoid cells following nylon fibre filtration. As previously stated, phospholipase C hydrolyses only the polar head groups of phospholipids while Wilkinson (1977) has suggested that the tail region of membrane Sphingomyelin may be important in extracellular interactions. Rheumatoid EA rosette forming cells which do not bear FacbRs appear to have an altered membrane lipid content; it is tempting to speculate that the FacbR bearing cell also has membrane lipid differences which are not detected by phospholipase C.

Attempts to correlate raised percentages of FacbR positive cells with disease activity in a cross-sectional study proved fruitless. No correlation was found between percentage Facb rosettes and plasma viscosity, ESR (Fig. 3.8) or thermographic index (Fig. 3.10). Patients with osteoarthritis and ankylosing spondylitis had normal percentage levels of Facb rosettes (Fig. 3.5); this observation could lead to the supposition that it is the auto-immune aspect of

RA rather than the inflammatory component which is responsible for the increased percentages of these cells. However percentage Facb rosettes also showed no correlation with the presence of either IgM or IgG antiglobulins (Figs. 3.6 and 3.7). It was noted, however, that Facb rosettes were increased particularly in early disease (< 2 years).

The lack of relationship between percentage Facb rosettes and the expression of the DRw4 antigen (Fig. 3.11) is perhaps not surprising. Early work, using L lymphocytes, described a DR positive, C3 receptor negative sub-group (Horwitz et al, 1978). It is now accepted that the majority (>90%) of non-cultured L cells lack a DR antigen (Lobo, 1981). The results obtained in this thesis (Fig. 3.11) indicate that there is no correlation between DRw4 positivity and FacbR expression implying that there is no membrane interaction between these molecules.

Rheumatoid FacbR positive lymphocytes, like those of healthy individuals, did not exhibit K or NK cell activity. Rheumatoid patients have been shown to exhibit normal or subnormal ADCC (Frøland, Natvig and Wisløff, 1975; McGill and Twinn, 1977; Panayi and Corrigall, 1977; Froebel et al, 1979; Penschow and MacKay, 1980). If FacbR positive cells were responsible for K cell activity, one might expect increased ADCC in rheumatoid peripheral blood. Similarly rheumatoid peripheral blood SCMC activity has also been described as normal (Penschow and MacKay, 1980; Panayi and Highton, 1980) or decreased (Burmester et al, 1978).

(vi) General Discussion

This discussion has so far been restricted to a discussion of the results obtained with FacbR positive cells and their possible relationship to L or third population lymphocytes. FacbR positive lymphocytes are a subpopulation of cells lacking conventional B and T cell surface markers but possessing high avidity FcγRs and thus resemble L cells. Perhaps the most surprising finding is that FacbR positive lymphocytes are present in low percentage numbers in healthy individuals and do not exhibit ADCC or SCMC; both of these observations oppose the published results for L and third population cells. It is therefore probable that L cells and FacbR positive lymphocytes are overlapping but not identical populations, the latter possibly being a subset of L cells which are not cytotoxic.

This short section will be assigned to an attempt to define a possible role for FacbR positive lymphocytes. Since the majority of work in this thesis is confined to surface receptor expression, cell characterisation and immunoglobulin binding specificity and not to the immune responsiveness of FacbR positive cells, this discussion will rely on published work using L lymphocytes.

Until very recently, little was known of the role of L lymphocytes in immune responses. In 1977, Horwitz presented evidence for cytotoxicity by L lymphocytes and postulated that these cells may have inducer/suppressor potential. Experimental evidence for the L lymphocyte as an accessory cell was provided by Carvahlo, Davis and Horwitz (1980). L cells could not replace monocytes in helping

B cells respond but they enhanced the blastogenic response of sensitised human lymphocytes to KLH-anti KLH complexes. Lobo (1981) has confirmed the accessory effect of L lymphocytes and shown that these cells also have suppressor activity. L lymphocytes, when added to peripheral blood mononuclear cells, are always stimulatory; this differs from the findings for T lymphocytes which can exert positive or negative responses depending on the ratio of T suppressor to T helper cells. Lobo (1981) also showed that culturing L lymphocytes for 48 hours or modulation with EA immune complexes induced potent suppressor activity. Addition of Con-A did not augment the observed suppression. Other workers have shown that nylon non-adherent cells (enriched for null and T cells) develop suppressor activity following incubation (Frøland and Natvig, 1973; Horwitz and Lobo, 1975). The existence of T_{γ} suppressor cells is well documented (Moretta, Webb, Grossi, Lydyard and Cooper, 1977) and the possible overlap of Tγ and L cells with respect to OKM1 positivity has been discussed previously (Reinherz et al, 1980; Kay and Horwitz, 1980). Hayward and colleagues (1978) have described the existence of a Tμ-derived suppressor population which is activated by Con-A and transition of Tγ to Tμ has been described (Pichler, Lum and Broder, 1978); however Con-A activated Tμ-derived suppressor cells are radioresistant (Hayward et al, 1978) while L suppressor and accessory functions are radiosensitive (Lobo, 1981). Lobo (1981) suggests that Tμ-derived suppressor cells may be necessary during the primary response while L lymphocytes may provide feedback control of the secondary immune response.

Percentage FacbR positive cells are raised following immunisation of sensitised individuals (Fig. 3.14). This observation may parallel

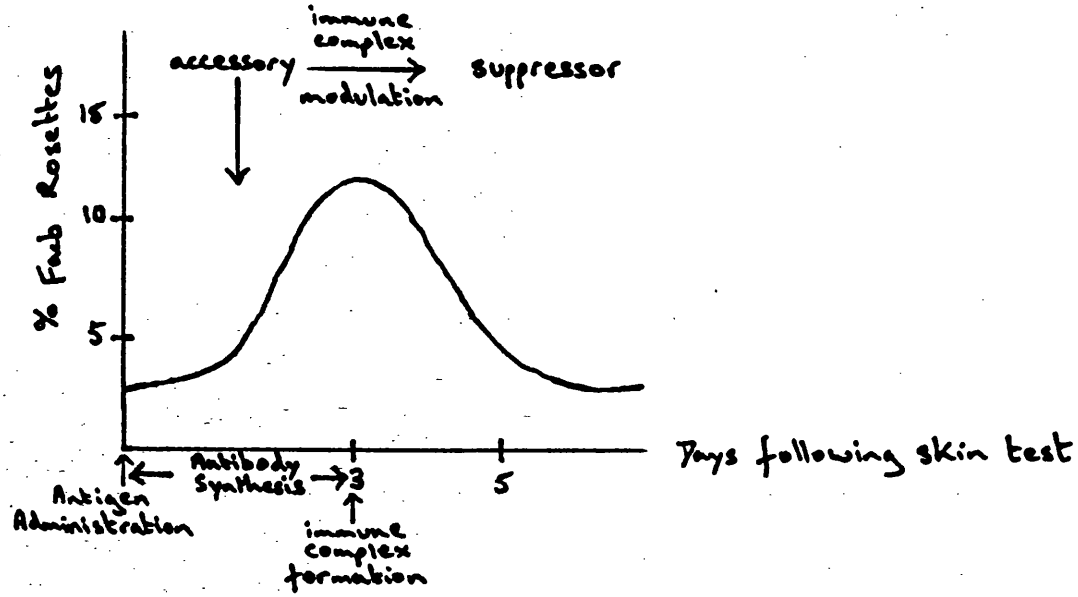
the findings of Carvahlo, Davis and Horwitz (1980). The increased percentages of FacbR positive cells may indicate recruitment of L accessory cells. Following B cell antibody production, immune complex modulation may induce L suppressor activity as described by Lobo (1981). By day 5, healthy immunised individuals show normal percentage levels of FacbRs; this may indicate that the FacbR is expressed or detectable only on accessory L lymphocytes and may also help to explain the lower numbers of FacbR cells measured in comparison with L or third population lymphocytes and the residual cytotoxic activity in Facb depleted mononuclear cell preparations (Figs. 6.1 and 6.2).

No skin testing was performed on rheumatoid individuals. If FacbRs are expressed only on L accessory cells and percentage FacbR positive cells are high in rheumatoid patients, it is possible that rheumatoid FacbR positive cells lack the capacity to suppress following immune complex modulation. This may be specific for Ig/Ig complexes. Alternatively the FacbR may be modified on rheumatoid cells such that the necessary interaction between the receptor and the immune complex does not occur. A schematic diagram is shown in figure 7.1. The idea that Facb expression is specific to accessroy function is further strengthened by the observations on cultured and mitogen stimulated mononuclear cells; FacbR expression decreased irrespective of whether mitogens were present (Figs. 5.7 and 5.8). Lobo (1981) described increased L suppressor activity following culture.

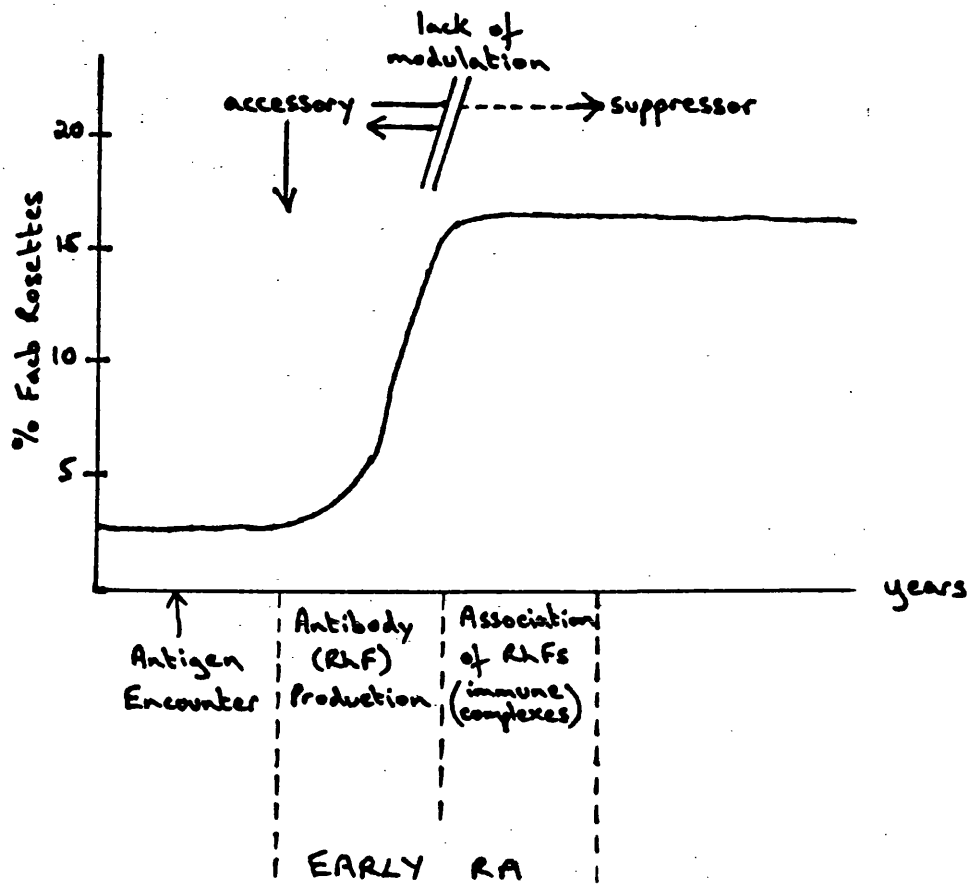
Raised percentages of FacbR positive lymphocytes are detected in patients with rheumatoid arthritis and appear to be particularly

FIGURE 7.1

(a) POSSIBLE EXPLANATION OF OBSERVED CHANGES IN FacbR POSITIVE CELLS FOLLOWING SKIN TESTING OF SENSITISED HEALTHY INDIVIDUALS



(b) SPECULATION ON OBSERVED HIGH Facb ROSETTES IN RHEUMATOID ARTHRITIS WITH RESPECT TO OBSERVED CHANGES IN SKIN TESTING (ABOVE)



high in early RA (< 2 years; Fig. 3.9 a). Abdou and co-workers (1981) described a reduced Con-A suppressor T cell activity in early active (< 3 months) rheumatoid patients and the presence of an anti-suppressor cell antibody in early RA. This may reflect the change from the primary response (Tμ-derived suppressor) to the secondary response (Tγ or L suppressor) as speculated by Lobo (1981) and may account for the very high percentage of FacbR positive cells observed in some patients with early disease. Meijer and associates (1980 a) have described the presence of an anti-Tμ antibody in active disease. No correlation was found between percentage Facb rosettes and disease activity (Figs. 3.8, 3.9b and 3.10). However no serial studies were performed and this may be necessary to detect individual changes.

Unlike T cells, L lymphocytes did not show differences in response dependent on the ratio of inducer/suppressor cells. The high levels of FacbR positive accessory cells in RA is difficult to interpret. The presence of immune complexes in rheumatoid sera would be expected to activate the L suppressor cell. It is possible that the immune complexes are of the wrong size or composition and are thus unavailable for binding. Binding characteristics of rheumatoid FacbR positive lymphocytes differed slightly from those of high avidity EA rosette forming cells (Fig. 4.2). No binding experiments were carried out on healthy cells due to practical difficulties. Perhaps a modification within the Fc binding site prevents the generation of suppressor activity following immune complex binding. However, both pre-washing of whole blood and trypsin treatment of rheumatoid mononuclear cells enhanced percentage Facb rosettes; this implies removal of

immune complexes or IgG from receptor sites. Also rheumatoid lymphocytes behave in a similar manner to healthy cells when cultured in that they lose FacbRs (Figs. 5.7 and 5.8). It would be interesting to know whether cultured rheumatoid FacbR positive cells exerted suppressor activity. The mitogen results (Table 6.5) provide very few clues. In the one rheumatoid lymphocyte population which was depleted of total EA rosettes (RA4), the PWM response was enhanced when compared with the sham depleted population; this enhancement was also seen in the Facb depleted population but to a lesser degree. This might suggest the removal of FacbR positive suppressor cells. Similar results were seen with one healthy individual (Healthy 1; Table 6.5). However the data is insufficient for further analysis.

From the data available it is tempting to speculate on the presence of a C3R negative accessory cell which can form rosettes with Facb sensitised erythrocytes. The presence of high percentages of these cells in rheumatoid patients is consistent with the continued production of IgM rheumatoid factor; however it is unclear from this data why immune complex modulation does not induce the loss of FacbRs and thereby possibly suppressor activity.

(vii) Suggestions for Further Studies

The low percentage numbers of FacbR positive cells in healthy individuals make studies of these cells difficult. It is obvious that a better purification technique is necessary since the time interval in isolation may affect the final result when using functional assays. The most useful method of purification would

be the development of an anti-FacbR antibody. Since high percentage numbers of Facb rosettes were detected in one rheumatoid patient with myeloid leukaemia, it may be useful to test other CML patients without RA for the presence of FacbRs. In the absence of a specific FacbR antibody, a FcγR antibody could be used to enrich for FacbR positive cells although this would still require a second Ficoll-Paque separation to deplete FacbR bearing cells.

Owing to the difficulties encountered using purified human lymphocytes, it may be beneficial to search for FacbR positive cells in other mammals. The presence of these cells during primary or secondary immune responses could be ascertained. This may give a clearer picture of the situation in man.

Direct measurements still need to be carried out on human cells. Thus it would be interesting to know whether FacbR positive cells form Ripley rosettes and are OKM1 positive. Also the speculation concerning the loss of FacbR with transition to suppressor activity needs to be investigated although this is not necessarily indicative of the in vivo situation.

The whole field of lymphocyte characterisation remains confused at present owing to the lack of specificity of the available markers. Since Facb rosette forming cells have many characteristics of null lymphocytes but do not have cytotoxic potential, it is possible that this technique defines a specific population. The function of that small population may be difficult to investigate.

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